UNIVERSIDAD COMPLUTENSE DE MADRID

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Departamento de Sanidad Animal

TESIS DOCTORAL

Estrategias para la mejora del diagnóstico serológico de la besnoitiosis bovina

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

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TESIS DOCTORAL

Dña. Paula García Lunar

Madrid, 2015

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CERTIFICAN:

Que la tesis doctoral titulada "Estrategias para la mejora del diagnóstico serológico de la besnoitiosis bovina" que presenta la Licenciada en Veterinaria Dña. Paula García Lunar ha sido realizada en las dependencias del Departamento de Sanidad Animal de la Facultad de Veterinaria, de la Universidad Complutense de Madrid bajo su supervisión y cumple todas las condiciones exigidas para optar al grado de Doctor por la Universidad Complutense de Madrid con Mención Europea.

De acuerdo con la normativa vigente, firmamos el presente certificado, autorizando su presentación como directores de la mencionada Tesis Doctoral.

En Madrid, a veintinueve de octubre de 2015

Fdo. Prof. Dra. Gema Álvarez García Fdo. Prof. Dr. Luis Miguel Ortega Mora

Y uno aprende

Después de un tiempo
uno aprende la sutil diferencia
entre sostener una mano
y encadenar un alma.
Y uno aprende
que el amor no significa recostarse
y una compañía no significa seguridad.
Y uno empieza a aprender...
que los besos no son contratos
y los regalos no son promesas.
Y uno empieza a aceptar sus derrotas
con la cabeza alta y los ojos abiertos.
Y uno empieza a construir
todos sus caminos en el hoy,
porque el terreno de mañana
es demasiado inseguro para planes...
y los futuros tienen una forma de caerse en la mitad.
Y después de un tiempo
uno aprende que, si es demasiado,
hasta el calorcito del sol quema.
Así que uno planta su propio jardín
y decora su propia alma,
en lugar de esperar que alguien le traiga flores.
Y uno aprende que realmente puede aguantar,
que uno realmente es fuerte,
que uno realmente vale.
Y con cada día uno aprende.

J. Luis Borges

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LISTADO DE ABREVIATURAS

1-DE	Electroforesis en una dimensión	One dimensional electrophoresis
2-DE	Electroforesis en dos dimensiones	Two dimensional electrophoresis
ADN/DNA	Ácido desoxirribonucleico	Desoxyribonucleic acid
AMA	Antígeno de membrana apical	Apical membrane antigen
ARN/RNA	Ácido ribonucleico	Ribonucleic acid
ARNm/RNAm	Ácido ribonucleico mensajero	Messenger ribonucleic acid
ARNt/RNAt	Ácido ribonucleico transferente	Transfer ribonucleic acid
ASB/BSA	Albúmina sérica bovina	Bovine serum albumin
ATP	Adenosin trifosfato	Adenosine triphosphate
B	Bradizoíto	Bradyzoite
BAG	Antígeno de bradizoíto	Bradyzoite antigen
Bb	*Besnoitia besnoiti* (prefijo)	*Besnoitia besnoiti* (prefix)
°C	Grados centígrados	Grades centigrades
CBB	Azul de coomassie brillante	Coomassie brilliant blue
cm	Centímetro	Centimeter
CV	Coeficiente de variación	Coefficient of variation
dpsc	Días post seroconversión	Days post seroconversion
DIGE	Electroforesis diferencial en gel	Difference gel electrophoresis
DO/OD	Densidad óptica	Optical densitiy
EDA	Análisis de datos extendidos	Extended data analysis
EEP/EPM	Encefalomielitis equina por protozoos	Equine protozoal myeloencephalitis
ELISA	Ensayo inmunoenzimático	Enzyme-linked immunosorbent assay
ENO	Enolasa	Enolase
Esp/Sp	Especificidad	Specificity
EST	Marcador de secuecia expresada	Expressed sequence tags
FDR	Falsa tasa de descubrimiento	False discovery rate
GA3PDH	Gliceraldehído 3-fosfato deshidrogenasa	Glyceraldehyde 3-phoshate dehydrogenase
H-E	Hematoxilina y eosina	Hematoxylin and eosin
Hb	Hemoglobina	Haemoglobin
HCA	Análisis jerárquico de agrupamientos	Hieralchical clustering analysis
HSP	Proteína de estrés térmico	Heat shock protein

Hyp	Proteína hipotética	Hypothetic protein
IA	Inseminación artificial	Artificial insemination
IC/CI	Intervalo de confianza	Confidence interval
ICW	Pared interna del quiste	Inner cyst wall
IDA	Antígeno inmunodominante	Immunodominant antigen
IEF	Enfoque isoeléctrico	Isoelectric focusing
IFI/IFAT	Inmunofluorescencia indirecta	Indirect immunofluorescence assay
IFN-γ	Interferón gamma	Interferon gamma
IRPC/RIPC	Índice relativo por cien	Relative index per cent
IHQ/IHC	Inmunohistoquímica	Inmunohistochemistry
ITS-1	Espacio de transcripción interna-1	Internal transcribed spacer-1
kDa	Kilodalton	Kilodalton
LDH	Lactato deshidrogenasa	Lactate dehydrogenase
MAB	Anticuerpo monoclonal	Monoclonal antibody
MALDI-TOF	Desorción/ionización láser asistida por matriz-tiempo de vuelo	Matrix assisted laser desorption/ionization-time of flight
Mb	Megabase	Megabase
MDH	Malato deshidrogenasa	Malate dehydrogenase
MIC	Proteína de micronemas	Microneme protein
Mm/MWS	Marcador de peso molecular	Molecular weight standard
MS	Espectrometría de Masas	Mass espectrometry
NAD	Nicotinamida adenina dinucleótido	Nicotinamide adenine dinucleotide
Nc	*Neospora caninum* (prefijo)	*Neospora caninm* (prefix)
nr	No reductor	Non-reducing
N-Z	Nueva Zelanda	New-Zealand
OCW	Pared externa del quiste	Outer cyst wall
OR	Odds ratio	Odds ratio
p	Putativa (sufijo)	Putative (sufix)
pb	Par de bases	Base pair
PCA	Análisis del componente principal	Principal component analysis
PCR	Reacción en cadena de la polimerasa	Polymerase chain reaction
PDI	Proteína disulfuro isomerasa	Protein disulfure isomerase
p*I*	Punto isoeléctrico	Isoelectric point
PMF	Huella peptídica	Peptide mass fingerprint

PNP	Purina nucleósido fosforilasa	Purine nucleoside phosphorilase
PP	Porcentaje de positividad	Per cent positiviy
r	Reductor	Reducing
ROC	Característica operativa del receptor	Receiver operating characteristic
RON	Proteína de cuello de roptrias	Rhoptry neck protein
ROP	Proteína de cuerpo de roptrias	Rhoptry bulb protein
RT	Temperatura ambiente	Room Temperature
SDS-PAGE	Electroforesis en gel de poliacrilamida con dodecilsulfato sódico	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Se	Sensibilidad	Sensitivity
SNC	Sistema nervioso central	Central nervous system
SOD	Superóxido dismutasa	Superoxide dismutase
spi/wpi	Semanas post infección	Weeks post infection
S/P	Ratio muestra/control positivo	Sample/positive control ratio
T	Taquizoíto	Tachyzoite
TEM	Microscopía electrónica de transmisión	Transmission electron microscopy
Tg	*Toxoplasma gondii* (prefijo)	*Toxoplasma gondii* (prefix)
WB	Western blot	Western blot

Capítulo I

Besnoitia besnoiti es un protozoo apicomplejo formador de quistes responsable de la besnoitiosis bovina, una enfermedad crónica y debilitante que origina importantes pérdidas económicas en el ganado bovino a nivel mundial. En la actualidad, no existen tratamientos ni vacunas disponibles y, por tanto, las medidas de control se deben basar en la detección de animales infectados para reducir la prevalencia en zonas endémicas y evitar la entrada de la enfermedad en zonas libres a través de la compra de animales infectados. Los principales retos en la investigación sobre este parásito son, por un lado, la mejora de las técnicas serológicas para realizar un diagnóstico preciso y, por otro lado, el empleo de las nuevas pruebas diagnósticas desarrolladas para determinar el impacto de la enfermedad mediante estudios de prevalencia e incidencia. Dada la rápida diseminación de la besnoitiosis bovina en Europa, se han desarrollado numerosas técnicas de diagnóstico serológico. Sin embargo, éstas no han sido validadas en estudios comparativos y, por ello, los datos epidemiológicos descritos por los diferentes laboratorios, no son comparables. Además, no se ha establecido una prueba de referencia, lo cual es de vital importancia para el establecimiento de un protocolo de diagnóstico común entre los países afectados. Por otra parte, se ha puesto de manifiesto la necesidad de mejorar la sensibilidad (Se) de las pruebas para detectar a los animales infectados durante la fase aguda, así como los infectados durante la fase crónica que presentan niveles bajos de anticuerpos, los cuales en diversas ocasiones se encuentran por debajo del punto de corte de las técnicas diagnósticas. Además, también es necesario mejorar su especificidad (Esp), ya que las actuales pruebas ELISA, que se emplean de forma rutinaria en el diagnóstico de la infección, pueden dar lugar a un elevado número de resultados falsos-positivos que pueden repercutir de forma negativa en la eficacia de los planes de control.

Por ello, en la presente Tesis Doctoral, se han validado por primera vez las técnicas de diagnóstico serológico que se utilizan de forma rutinaria en Europa y se ha establecido un criterio de diagnóstico común entre los países que utilizan habitualmente estas técnicas (Objetivo 1). En este trabajo, se ha demostrado la utilidad de los ELISAs en el diagnóstico y en los estudios epidemiológicos debido a las buenas características diagnósticas

observadas. Sin embargo, el Western blot ha mostrado características diagnósticas mejores y, por ello, se ha considerado la prueba de referencia. Por tanto, se recomienda su empleo como prueba confirmatoria en determinadas ocasiones, como en el caso de resultados dudosos, para determinar el estatus sanitario de las nuevas incorporaciones en rebaños libres de la enfermedad y para el diagnóstico de los animales valiosos antes de llevar a cabo un sacrificio selectivo.

Sin embargo, estudios recientes han puesto de manifiesto la existencia de un número elevado de resultados falsos-positivos mediante la prueba ELISA, lo cual puede comprometer notablemente los planes de control. Por ello, en el Objetivo 2, se ha investigado su origen. Para ello, se han estudiado las reacciones cruzadas serológicas entre antígenos del taquizoíto de *B. besnoiti*, y anticuerpos específicos anti-*N. caninum* y/o anti-*Sarcocystis* spp., ya que son parásitos filogenéticamente cercanos y sus infecciones son muy prevalentes en el ganado bovino a nivel mundial. Este trabajo ha puesto de manifiesto que los resultados falsos-positivos obtenidos con la prueba BbSALUVET ELISA 1.0, están asociados no sólo a la presencia de anticuerpos específicos anti-*Sarcocystis* spp. y anti-*N. caninum*, sino también a un elevado nivel de anticuerpos frente a ambos. Además, se ha puesto de manifiesto la importancia de incluir un número apropiado de sueros procedentes de animales seropositivos frente a *Sarcocystis* spp. y/o *N. caninum*, que además presenten niveles elevados de anticuerpos frente a ambos parásitos en el proceso de validación de las técnicas serológicas de la besnoitiosis bovina.

Debido a las limitaciones de las técnicas serológicas anteriormente descritas, el Objetivo 3 de la presente Tesis Doctoral se ha centrado en la identificación de nuevas dianas diagnósticas para mejorar la Se y Esp de las pruebas. Para ello, en primer lugar, mediante dos abordajes proteómicos, se ha intentado identificar antígenos específicos del taquizoíto de *B. besnoiti* (Sub-objetivos 3.1 y 3.2). Estos trabajos han permitido describir el proteoma y el inmunoma del estadio de taquizoíto de *B. besnoiti,* por primera vez, y se ha observado que la mayoría de las manchas proteicas inmunogénicas están localizadas entre 37 y 50 kDa y en la zona ácida de un gradiente de pH 3-10. Además, el proteoma e inmunoma de *B. tarandi* fue similar al descrito en *B. besnoiti*, si bien se han podido

detectar diferencias en abundancia de proteínas entre ambas especies. En estos estudios, se han identificado, por primera vez, 17 proteínas de *B. besnoiti* y *B. tarandi* abundantes y/o inmunogénicas. Desafortunadamente, todas ellas actúan en procesos conservados en los parásitos apicomplejos. En particular, cabe destacar que la mayoría se corresponde con proteínas del metabolismo, proteínas de estrés térmico y proteínas involucradas en la invasión de la célula hospedadora. Además, se han identificado seis proteínas más abundantes en el taquizoíto de *B. besnoiti* (lactato deshidrogenasa (LDH), proteína de estrés térmico (HSP) 90, purina nucleósido fosforilasa y tres proteínas hipotéticas) y seis más abundantes en el taquizoíto de *B. tarandi* (gliceraldehído 3-fosfato deshidrogenasa (G3PDH), LDH, proteína disulfuro isomerasa (PDI), proteína de la caperuza del ARNm y dos proteínas hipotéticas). Por otra parte, se han detectado 25 manchas proteicas implicadas en las reacciones cruzadas que se producen entre los anticuerpos específicos anti-*N. caninum* y el extracto del taquizoíto de *B. besnoiti,* de las cuales se pudieron identificar la fructosa1,6-bisfosfatasa aldolasa, enolasa (ENO), HSP60, HSP90 y la actina. Desafortunadamente, en ninguno de los estudios proteómicos llevados a cabo, se han podido identificar dianas diagnósticas, ya que todas las proteínas identificadas corresponden a proteínas conservadas entre protozoos.

Además, se han obtenido ocho anticuerpos monoclonales (MABs) frente a un extracto total y un extracto enriquecido en proteínas de membrana del taquizoíto de *B. besnoiti* (Sub-objetivo 3.3). Estos MABs han sido caracterizados en base a la localización de los epítopos reconocidos mediante microscopía confocal y microscopía electrónica de transmisión (TEM). Además se ha valorado su especificidad de género, especie y estadio mediante el estudio de las reacciones cruzadas con el estadio de taquizoíto de *B. tarandi, N. caninum, T. gondii,* con el estadio de cistozoíto de *Sarcocystis* spp., así como con el estadio de bradizoíto de *B. besnoiti*. Los MABs útiles para el diagnóstico deben reconocer antígenos inmunodominantes y específicos de *B. besnoiti.* Por ello, los MABs específicos del taquizoítos de *Besnoitia* spp. 2.G.A, 2.A.12 y 2.G.4, junto con los MABs que reconocen el extremo apical (1.17.8 y 8.9.2) y la superficie del taquizoíto (3.10.8 y 5.5.11) podrían ser buenos candidatos con fines diagnósticos.

Finalmente, se ha obtenido un extracto de taquizoítos liofilizados de *B. besnoiti* y se ha empleado para desarrollar una nueva prueba ELISA (BbSALUVET ELISA 2.0) (Sub-objetivo 3.4). En base a los resultados obtenidos en el Objetivo 2, esta prueba se ha validado usando un alto número de sueros con un resultado de ELISA falso-positivo y falso-negativo. Además, las características diagnósticas se han comparado con las observadas cuando se emplea una prueba *in house* (APure-BbELISA) basada en un extracto enriquecido en proteínas de membrana del taquizoíto y un ensayo comercial (PrioCHECK Besnoitia Ab 2.0) previamente desarrolladas. Cabe destacar que BbSALUVET ELISA 2.0 y APure-BbELISA han mostrado características diagnósticas similares y, por tanto, pueden ser utilizadas indistintamente con fines diagnósticos sin la necesidad de emplear el Western blot como prueba confirmatoria. PrioCHECK Besnoitia Ab 2.0 ha mostrado valores excelentes de Esp, si bien los valores de Se observados fueron inferiores al 90%. Por lo tanto, en este caso, se recomienda el empleo del Western blot para evitar resultados de ELISA falsos-negativos. Por otra parte, se ha confirmado la utilidad adicional de la nueva prueba BbSALUVET ELISA 2.0 para la detección de la infección por *Besnoitia* spp. en rumiantes silvestres y, por tanto, puede resultar útil para detectar anticuerpos específicos anti-*Besnoitia* spp. en estas especies. Finalmente, es la primera vez que se emplean taquizoítos liofilizados de *B. besnoiti* para el diagnóstico de *Besnoitia* spp. Su producción fácil y escalable, junto con los buenos resultados obtenidos, hacen que sea un extracto con fines comerciales muy atractivo y, por ello, la prueba ELISA basada en este extracto antigénico se ha protegido mediante una patente.

Besnoitia besnoiti is a cyst forming intracellular parasite that causes bovine besnoitiosis, a chronic and debilitating disease in cattle responsible for severe economic losses. Unfortunately, there are no vaccines or treatments available and disease control relies solely on management measures coupled with diagnosis. The two main challenges in the field of *B. besnoiti* research nowadays are: the improvement of serological tools for an accurate diagnosis of bovine besnoitiosis, and the use of these techniques in prevalence and incidence studies to determine the impact of the disease in affected countries. Due to the rapid spread of bovine besnoitiosis in Europe, many serological assays have been developed to date. Unfortunately, these tests have never been validated in comparative studies. Thus, the epidemiological data reported in different laboratories are not comparable. In addition, a gold standard test for bovine besnoitiosis, which is mandatory for establishing common control procedures among affected countries, has never been accepted. Moreover, recent studies have shown that the serological assays developed so far may show low sensitivity (Se) for detecting *B. besnoiti* acutely infected as well as *B. besnoiti* chronically infected cattle showing low anti-*B. besnoiti* antibody levels. Furthermore, the existence of false-positive reactors has been also reported using ELISA and IFAT tests, which may be due to the presence of cross-reactive antigens between *B. besnoiti* and other Sarcocystidae parasites.

In the present Doctoral Thesis, the techniques that are routinely employed for the diagnosis of bovine besnoitiosis in Europe were standardized and a common diagnostic procedure among affected countries was established for the first time (Objective 1). Based on the results obtained in this study, all ELISAs evaluated performed well and are useful tests for both diagnosis and epidemiological studies. However, Western blot performed better and was considered as the gold standard. Therefore, it may be recommended as a confirmatory assay under certain conditions, such as ambiguous results, cattle prior to entry to herds free of the disease and valuable animals prior to a selective culling.

However, recent studies have reported the existence of a high number of false-positive results using ELISA tests, which may notably compromise control programs and surveillance. Therefore, throughout Objective 2, the origin of the false-positive ELISA

results was investigated. In particular, cross-reactions between anti-*Sarcocystis* spp. and anti-*N. caninum* specific antibodies and *B. besnoiti* antigens were studied, since both infections are highly prevalent in cattle worldwide. Interestingly, the results revealed that *B. besnoiti* false-positive ELISA results were associated not only to the presence of anti-*Sarcocystis* spp. and anti-*N. caninum* specific antibodies, but also to high antibody levels against them. Thus, a more appropriate sera panel to be employed in future assays of validation was established, and it should comprise sera showing high levels of anti-*Sarcocystis* spp. and anti-*N. caninum* specific antibodies.

Due to the limitations of the serological assays previously mentioned, Objective 3 of the present Doctoral Thesis focused on the identification of new diagnostic targets in order to improve the Se and specificity (Sp) of the serological techniques already developed. For this purpose, first, two proteomic studies were carried out in order to identify valuable targets for a specific diagnosis (Sub-objectives 3.1 and 3.2). These studies allowed the description of *B. besnoiti* tachyzoite proteome and immunome and demonstrated that the majority of the abundant spots and antigenic spots were located between 37 and 50 kDa and in the acidic range of the pH gradient 3-10. In addition, *B. tarandi* showed very similar proteome and immunome profiles. However, differences in protein abundance between the two species studied were determined for the first time. Within these studies, 17 abundant and/or immunogenic *B. besnoiti* and *B. tarandi* proteins were identified for the first time. Unfortunately, all proteins identified were mainly related to metabolism, stress response and host cell invasion, and thus, were conserved among apicomplexan parasites. In particular, six up-regulated *B. besnoiti* proteins (lactate dehydrogenase (LDH), heat shock protein (HSP) 90, purine nucleoside phosphorylase and 3 hypothetical proteins) and six up-regulated *B. tarandi* proteins (glyceraldehyde 3-phosphate dehydrogenase (G3PDH), LDH, PDI, mRNA decapping protein and 2 hypothetical proteins) were successfully identified. In addition, up to 25 *B. besnoiti* cross-reacting spots were recognized by pool sera from *N. caninum* infected cattle, which may be responsible for the serological cross-reactions previously observed in Objective 2. Among these spots, fructose-1,6-bisphosphate aldolase, enolase (ENO),

HSP60, HSP90, and actin were identified. Unfortunately, none of these studies has led to the identification of potential diagnostic targets, since all identified antigens were conserved proteins among apicomplexan parasites.

Moreover, eight monoclonal antibodies (MABs) were developed against a whole- and a membrane enriched- *B. besnoiti* tachyzoite extract that may also be useful for *B. besnoiti* biology studies (Sub-objective 3.3). Co-localization and transmission electron microscopy (TEM) studies were carried out and in order to verify the genus- species- and stage- Sp cross-reactions with the tachyzoite stage of the closely related protozoan *B. tarandi, Neospora caninum, Toxoplasma gondii,* with the cystozoite stage of *Sarcocystis* spp. and with the bradyzoite stage of *B. besnoiti* were investigated using Western blot. Ideally, MABs useful for diagnostic purposes require the recognition of *B. besnoiti* specific and immunodominant antigens. Thus, the *Besnoitia* spp. tachyzoite specific MABs 2.G.A, 2.A.12 and 2.G.4, together with those recognizing the apical tip of the tachyzoite (1.17.8 and 8.9.2), and the surface of the tachyzoites (3.10.8 and 5.5.11) arise as promising diagnostic candidates.

Finally, a *B. besnoiti* lyophilized tachyzoite extract was obtained and employed for the development of a new ELISA test (BbSALUVET ELISA 2.0) (Sub-objective 3.4). Based on the results derived from Objective 2, this assay was evaluated under the worst-case scenario by using a high number of false-positive and false-negative ELISA reactors. Its performance was compared with an in house ELISA (APure-BbELISA) based on an enriched membrane extract and a commercial ELISA assay (PrioCHECK Besnoitia Ab2.0). Since BbSALUVET ELISA 2.0 performance was excellent and comparable to that obtained with APure-BbELISA, both tests can be equally employed with diagnostic purposes without the need of Western blot as a confirmatory assay. PrioCHECK Besnoitia Ab2.0 showed excellent Sp but lower Se values. Therefore, the employment of an additional Western blot test may be useful to avoid false negative results in valuable samples. The additional usefulness of the new assay for the diagnosis of *Besnoitia* spp. infection in wild ruminants was also confirmed. Therefore it can be employed to further study the epidemiology of the *Besnoitia* species affecting wild ruminants. Moreover,

lyophilized whole tachyzoite antigen has never been employed for the diagnosis of *Besnoitia* spp. infections in ELISA tests. Notably, the excellent performance observed, the easy and scalable production of the antigenic extract together with the good initial results of precision using different batches of antigen makes it an attractive extract for future market exploitation and the ELISA based on this extract has already been protected under intellectual property.

Capítulo II

1. Introducción histórica

La besnoitiosis bovina, originada por el protozoo apicomplejo formador de quistes *Besnoitia besnoiti*, es una enfermedad crónica y debilitante que se caracteriza por la presencia de lesiones en piel y alteraciones sistémicas. Desde su origen ha recibido diversas denominaciones como globidiosis, sarcosporidiosis cutánea bovina o elefantiasis. El primer caso de besnoitiosis bovina en el mundo fue descrito en el sur de Francia en 1884 por Cadéac (Cadéac, 1884), que denominó a la enfermedad como "l'élephantiasis et l'anasarque du boeuf". Sin embargo, se sospecha que la enfermedad tuvo su origen en África, ya que en Portugal se detectaron animales infectados entre el ganado importado desde Angola (Leitão, 1949). En 1912, Besnoit y Robin describieron la presencia de diversos casos en la zona del Pirineo Francés, y denominaron al agente etiológico *Sarcocystis* spp. (Besnoit y Robin, 1912). Sin embargo, hasta ese momento no se había descrito ninguna enfermedad similar en el ganado bovino y Marotel (Marotel, 1912) sugirió el término *Sarcocystis besnoiti* para referirse a su agente etiológico. En 1916, Franco y Borges describieron la presencia de casos clínicos de la enfermedad en Portugal, en la zona del Alentejo. Fue en este trabajo cuando, de forma definitiva, se denominó al agente etiológico como *Besnoitia besnoiti* y se introdujo el término besnoitiosis para referirse a la enfermedad producida por este parásito (Franco y Borges, 1916).

Desde la primera descripción de la enfermedad en Sudáfrica (Hofmeyr, 1945), y hasta los años setenta, se obtuvieron en esta región los primeros aislados de *B. besnoiti* que procedían de ungulados infectados naturalmente, tanto domésticos (ganado bovino), como silvestres (impala y ñu). Este hecho permitió realizar las primeras infecciones experimentales en bovinos, roedores y lagomorfos, y permitió además dilucidar algunos aspectos relacionados con la transmisión de la enfermedad y su patogenia (Pols, 1960; Bigalke et al., 1967; Bigalke, 1968; Basson et al., 1970). En esos años, se describieron la vía de transmisión horizontal por contacto directo entre animales infectados y sanos y el papel de los vectores mecánicos en la diseminación de la enfermedad. Si bien los resultados obtenidos en estos trabajos no son comparables debido a las diferentes condiciones utilizadas, todos los autores coincidieron en señalar la dificultad de inducir

signos clínicos en animales infectados experimentalmente. Paralelamente, Basson et al. (1965, 1970) y McCully et al. (1966) llevaron a cabo los primeros trabajos de patogénesis y distribución intraorgánica del parásito. En sus estudios, describieron de forma detallada los signos clínicos de la enfermedad durante las fases aguda y crónica en infecciones naturales y experimentales, así como la distribución intraorgánica de los quistes tisulares, destacando la descripción de su presencia en el sistema cardiovascular (McCully et al., 1966). Curiosamente, se pudo observar que los animales infectados desarrollaban una respuesta inmunitaria protectora frente a la enfermedad (Bigalke, 1968). Además, se observó que los aislados procedentes de ñues parecían menos virulentos que los aislados de origen bovino, ya que producían signos clínicos y lesiones más leves en esta última especie. Estos estudios sentaron las bases para el posterior desarrollo de una vacuna viva basada en un aislado de baja virulencia procedente del ñu, que lograba evitar el desarrollo de signos clínicos en los animales vacunados durante un periodo de hasta cuatro años (Bigalke et al., 1974).

A partir de los años setenta, hay que destacar el trabajo realizado por Kumi-Diaka et al. (1981), donde describieron las lesiones asociadas a la multiplicación del parásito en el aparato genital de toros infectados que cursaban infertilidad. Por otra parte, los trabajos llevados a cabo en Israel se centraron en profundizar en la distribución intraorgánica de quistes tisulares del parásito mediante estudios histopatológicos en el aparato genital de hembras y machos infectados (Neuman, 1972; Nobel et al., 1977, 1981), así como en realizar los primeros estudios serológicos que pusieron de manifiesto la importancia de la enfermedad en este país (Neuman, 1972; Frank et al., 1977; Goldman y Pipano, 1983). Sin embargo, fue a finales de los años ochenta cuando, tras el mantenimiento de un aislado de *B. besnoiti* procedente de un toro naturalmente infectado en cultivo celular (Neuman, 1974), fue posible la realización de los primeros estudios morfológicos con TEM, así como los primeros ensayos *in vitro* e *in vivo* para evaluar posibles tratamientos frente a la infección (Shkap et al., 1985, 1988). Sin embargo, los estudios más relevantes incluyeron la valoración de una vacuna basada en un aislado avirulento de origen bovino, en diferentes especies animales (Shkap, 1986; Shkap et al., 1987). Esta vacuna, cuya

seguridad todavía se desconoce, se utiliza en la actualidad de forma rutinaria en este país para evitar el desarrollo de signos clínicos en los sementales.

En relación a Europa, desde su primera descripción en los Pirineos y en Portugal (Cadéac, 1884; Franco y Borges, 1916) hasta finales de los años noventa, no se le prestó mucha atención a la enfermedad. Sin embargo, debido al número creciente de casos descritos en España (Juste et al., 1990; Fernández-García et al., 2009b), en Portugal (Cortes et al., 2004, 2005, 2006a, b, c) y en Francia (Alzieu, 2007), junto con la descripción de nuevos brotes en otros países cercanos como Alemania, Croacia, Hungría, Italia y Suiza, la besnoitiosis bovina está considerada en la actualidad una enfermedad re-emergente en Europa (EFSA, 2010; revisado por Cortes et al., 2014). Por ello, durante las últimas décadas se han desarrollado numerosas técnicas de diagnóstico, con el fin de realizar adecuados planes de control, y se ha profundizado en la cronobiología de la infección durante las fases aguda y crónica en condiciones naturales (Frey et al., 2013a, b; Langenmayer et al., 2015a, b). En estos últimos años, el conocimiento de la prevalencia e incidencia de la enfermedad en zonas endémicas ha aumentado considerablemente. Sin embargo, quedan aún muchas incógnitas por esclarecer relacionadas, por un lado, con la biología del parásito y con la epidemiología de la enfermedad y, por otro, con la respuesta inmunitaria del hospedador frente a la infección. Finalmente, el desarrollo de tratamientos y/o vacunas eficaces, hoy por hoy, es una prioridad para lograr el control de la enfermedad.

2. *Besnoitia besnoiti*

2.1. Taxonomía y espectro de hospedadores

Besnoitia besnoiti es un protozoo intracelular obligado perteneciente al filo Apicomplexa, familia Sarcocystidae y subfamilia Toxoplasmatinae que incluye otros géneros de parásitos heteroxenos formadores de quistes como *Toxoplasma, Neospora* y *Hammondia* (Tenter et al., 2002). Se conocen hasta el momento diez especies dentro del género *Besnoitia* (*B. besnoiti, B. bennetti, B. caprae, B. tarandi, B. akadoni, B. jellisoni, B.*

darlingi, B. neotomofelis, B. oryctofelisi, y *B. wallacei*) (Dubey y Yabsley, 2010), si bien sólo *B. besnoiti*, *B. bennetti, B. caprae* y *B. tarandi* afectan a ungulados (Dubey et al., 2003a, 2004, 2005) (Tabla 1).

Tabla 1. Especies del género *Besnoitia*.

[1] Roedor; [2] Reptil; [3] Marsupial; [4] Lagomorfo.
* Se sospecha que el hospedador definitivo es el gato. En concreto, fue sugerido por Peteshev et al. (1974), aunque otros autores no han podido reproducir sus resultados.

Especie	Hospedador intermediario	Hospedador definitivo	Distribución geográfica	Primera descripción
B. besnoiti	Bovino, antílope, impala y ñu azul	Desconocido *	África, Asia, Europa y Venezuela	Besnoit y Robin, 1912
B. bennetti	Burro, caballo y cebra	Desconocido	África, EE.UU. y Francia	Bennett, 1927
B. caprae	Cabra	Desconocido	Irán y Kenia	Cheema y Toofanian, 1979
B. tarandi	Buey almizclero, caribú, ciervo-mula y reno	Desconocido	Canadá, EE.UU., Finlandia, Rusia y Suecia	Hadween, 1922
B. akodoni	*Akodon montensis* [1]	Desconocido	EE.UU.	Dubey et al., 2003c
B. jellisoni	*Dipodomys* spp. [1] *Peromyscus maniculatus* [1]	Desconocido	EE.UU.	Frenkel, 1953
B. darlingi	*Ameiva* [2], *Basiliscus* [2] y *Didelphis marsupialis* [3]	Gato	EE.UU. y Panamá	Darling, 1910
B. neotomofelis	*Neotoma micropus* [1]	Gato	EEUU	Dubey y Yabsley, 2010
B. oryctofelisi	*Oryctolagus cuniculus* [4]	Gato	Argentina y EE.UU.	Dubey y Lindsay, 2003
B. wallacei	*Rattus* [1]	Gato	Australia, Hawái, Japón y Kenia	Wallace y Frenkel, 1975

La infección por *B. besnoiti* fue descrita, por primera vez, en el ganado bovino en Francia (Besnoit y Robin, 1912) y, varias décadas más tarde, en el ñu y el impala en Sudáfrica (McCully et al., 1966). Desde los años sesenta a los ochenta se obtuvieron en Sudáfrica los primeros aislados procedentes de ungulados infectados naturalmente (Bigalke et al., 1967; Bigalke, 1968; Basson et al., 1970). Desafortunadamente, en la actualidad estos aislados africanos ya no están disponibles. Sin embargo, durante los últimos años se han obtenido y descrito numerosos aislados de origen bovino a partir de biopsias de animales crónicamente infectados (Bb-Israel, Bb1Evora03, Bb2Evora03, Bb Spain1, Bb-GER1, Bb-Italy1, Bb-IPZ-1-CH, Bb-IPZ-2-CH, Bb-IPZ-3-CH, Bb-French) (Dubey et al., 2003a; Cortes et al., 2006c; Fernández-García et al., 2009b; Schares et al., 2009; Gentile et al., 2012; Basso et al., 2013; Liénard et al., 2013). Muchos de estos aislados se ha demostrado que presentan una identidad genética del 100% en las secuencias ribosomales 18S e ITS-1 (Schares et al., 2009). Sin embargo, se desconoce si presentan variaciones en su virulencia.

La otra especie incluida dentro del género *Besnoitia* que afecta a grandes rumiantes, *B. tarandi*, se describió originariamente en el reno (*Rangifer tarandus tarandus*) y en el caribú (*Rangifer tarandus caribou*) en Alaska (Hadween, 1922). En los pequeños rumiantes, en concreto en el ganado caprino, se han descrito infecciones similares que cursan con los signos clínicos típicos de la besnoitiosis bovina tanto en Irán como en Kenia, lugares donde la enfermedad se considera endémica y ha sido atribuida a la especie *B. caprae* (Cheema y Toofanian, 1979; Bwangamoi et al., 1989; revisado por Olias et al., 2011). En relación a la besnoitiosis equina, causada por *B. bennetti,* estuvo en un principio limitada a burros y caballos, existiendo una descripción puntual en una cebra africana (Pols, 1960; Bigalke, 1968; Bigalke y Prozesky, 1994). Sin embargo, diversos estudios han demostrado que actualmente es una enfermedad emergente en burros de EE.UU. (Dubey et al., 2005; Elsheikha et al., 2005; revisado por Olias et al., 2011; Ness et al., 2014).

Numerosos trabajos han puesto en cuestión la diferenciación específica dentro del género *Besnoitia* entre las especies que afectan a ungulados. Esto se debe, principalmente,

al desconocimiento de la identidad del hospedador definitivo en todas ellas, la ausencia de diferencias ultraestructurales entre *B. besnoiti, B. tarandi* y *B. bennetti* (Dubey et al., 2003a, 2004, 2005), las intensas reacciones cruzadas serológicas observadas entre *B. besnoiti* y *B. tarandi* (Gutiérrez-Expósito et al., 2012), así como entre *B. besnoiti* y *B. bennetti* (Ness et al., 2012) y a la similitud existente entre estas infecciones en relación a los signos clínicos y lesiones que desarrollan los animales infectados. Además, diversos estudios moleculares han demostrado que estas especies están muy relacionadas entre sí y que poseen idénticas regiones ITS-1, 18S y 5,8S del ARNr y tan sólo una inserción de 2pb en el segmento 28S diferencia *B. tarandi* de *B. besnoiti* (Schares et al., 2009; Ellis et al., 2000; revisado por Olias et al., 2011). De hecho, la única técnica de caracterización genética disponible en la actualidad es el análisis por microsatélites que ha permitido establecer algunas diferencias entre *B. besnoiti, B. tarandi* y *B. bennetti* (Madubata et al., 2012). Sin embargo, estas especies de *Besnoitia*, presentan importantes diferencias. En primer lugar, se han descrito en diferentes hospedadores intermediarios (Pols, 1960; Njenga et al., 1993; Nganga et al., 1994; Dubey et al., 2003a, 2004, 2005). Además, han mostrado diferente capacidad para infectar animales de laboratorio (Njenga et al., 1993; Nganga et al., 1994; Dubey et al., 2003a, 2004, 2005; Oryan et al., 2010). De hecho, una gran variedad de infecciones experimentales han demostrado que los gerbos y los conejos son susceptibles a la infección por *B. besnoiti* pero no a la infección por *B. tarandi* (Pols, 1960; Neuman, 1962; Bigalke, 1968; Shkap et al., 1987; Dubey et al., 2004; Basso et al., 2011).

2.2. Morfología y ciclo biológico

Se conocen dos estadios diferentes en el ciclo biológico de *B. besnoiti:* los taquizoítos (endozoítos) y los bradizoítos (cistozoítos), desarrollándose ambos en el hospedador intermediario (Figura 1). Hasta el momento no se han podido identificar los ooquistes de *B. besnoiti*. Estos serían eliminados por el hospedador definitivo y contendrían en su interior los esporozoítos, una de las fases infectantes para el hospedador intermediario. Sin embargo, se piensa que puedan ser similares a los ya descritos para otras especies de

Besnoitia (*B. darlingi, B. neotomofelis, B. oryctofelisi* o *B. wallacei*) en las que los ooquistes se eliminan sin esporular con las heces del hospedador definitivo, esporulando en el medio ambiente cuando las condiciones de temperatura y humedad son adecuadas. En estas especies, los ooquistes presentan un tamaño aproximado de 10-14 por 10-13 µm y contienen en su interior dos esporoquistes con cuatro esporozoítos cada uno (Dubey et al., 2003b, Dubey y Yabsley, 2010).

Los taquizoítos invaden las células endoteliales de los vasos sanguíneos, aunque también pueden multiplicarse en los monocitos, neutrófilos y hepatocitos (Pols, 1954; Schares et al., 2009; Langenmayer et al., 2015b). Tienen un tamaño aproximado de 6-7,5 por 2,5-3,9 µm (Reis et al., 2006) y forma ovoide o de media luna, con un extremo anterior acabado en punta y un extremo posterior redondeado. Se han encontrado tanto libres en el citoplasma de la célula hospedadora, como rodeados por una vacuola parasitófora formada por una sola membrana (Langenmayer et al., 2015b). Su ultraestructura se parece a la ya descrita para otros protozoos apicomplejos (Dubey et al., 1998; Speer et al., 1999). Están rodeados por una película formada por tres capas: la membrana externa (plasmalema), que rodea la superficie del taquizoíto; y una doble capa interna, que forma el complejo de membrana interno (Langenmayer et al., 2015b) (Figura 1). Presentan organelas típicas de la célula eucariota (núcleo, retículo endoplasmático, aparato de Golgi y mitocondria), así como otras organelas (apicoplasto, micronemas, roptrias y gránulos densos) y estructuras (anillos polares anterior y posterior, conoide, plasmalema y complejo interno de membrana) propias del filo Apicomplexa. En los taquizoítos, los gránulos de amilopectina se detectan de forma esporádica y el núcleo se encuentra situado en la mitad o en el tercio posterior del parásito. Se piensa que los taquizoítos invaden la célula hospedadora de forma similar a como lo hacen otros Toxoplasmatinae, con una secuencia de procesos incluidos en lo que se denomina ciclo lítico, y que incluyen la adhesión e invasión por parte de los taquizoítos a la célula, su multiplicación intracelular y, finalmente, su liberación, tras la lisis celular, en un proceso llamado egresión (revisado por Dubey y Lindsay, 1996; Hemphill et al., 1996; revisado por Hemphill et al., 2006).

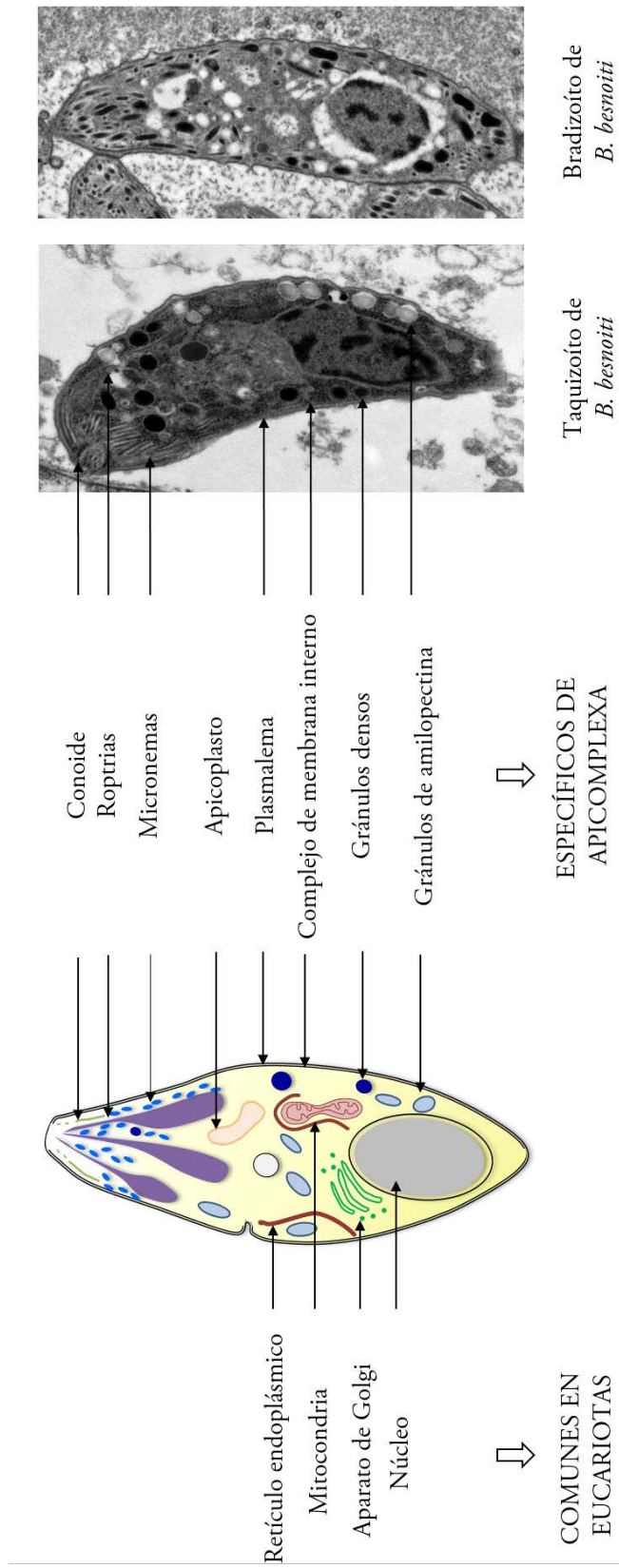
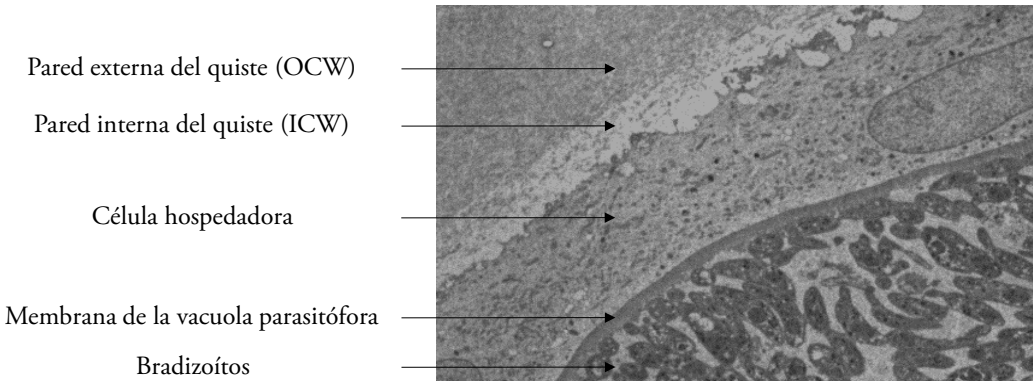


Figura 1. Morfología del taquizoíto y bradizoíto de *B. besnoiti* (derecha: imágenes obtenidas con TEM).

Por su parte, los bradizoítos están contenidos en el interior de quistes tisulares, que se localizan en el tejido conjuntivo del hospedador intermediario, principalmente en la piel y en las mucosas y, en menor medida, en otras localizaciones como tendones, tejido vascular, esclerótica, tracto respiratorio superior, testículo y epidídimo en los machos y vestíbulo vaginal en las hembras (Basso et al., 2013; Frey et al., 2013a, b). A diferencia de otros apicomplejos como *N. caninum* y *T. gondii,* en *B. besnoiti* no existe información sobre el proceso de cistogénesis. Sin embargo, se ha señalado que los quistes tisulares aparecen de forma sincrónica y que su tamaño aumenta a medida que progresa la infección (Bigalke, 1968). Así, a los seis días post seroconversión (dpsc) miden en torno a 30 µm, mientras que a los 73 dpsc pueden superar los 400 µm. Al contrario que en otros apicomplejos similares como *N. caninum* y *T. gondii,* la pared del quiste, que rodea la célula hospedadora, está formada por dos capas: una capa externa (OCW) que contiene múltiples fibras de colágeno entrelazadas y una capa interna (ICW), formada por proteoglicanos y extensiones filamentosas que se proyectan hacia la membrana externa (Langenmayer et al., 2015b) (Figura 2). El citoplasma de la célula hospedadora aparece deformado y contiene el núcleo y las diferentes organelas celulares. La vacuola parasitófora está inmediatamente por debajo del citoplasma celular y se encuentra delineada por una membrana de estructura granular. En su interior se encuentran numerosos bradizoítos en diferente estado evolutivo (Langenmayer et al., 2015b) (Figuras 1 y 2). El tamaño de éstos es de 6,0-7,5 por 1,9-2,3 µm (Dubey et al., 2003a) y en ellos encontramos las mismas organelas que en los taquizoítos. Sin embargo, poseen una forma más alargada y en su citoplasma se observa un mayor número de gránulos de amilopectina. Aunque los bradizoítos de las diferentes especies de *Besnoitia* poseen muchos rasgos comunes (Heydorn et al., 1984; Njenga et al., 1995; Dubey y Lindsay, 2003; Dubey et al., 2003a, 2004, 2005) y, de hecho, no se han encontrado diferencias ultraestructurales entre *B. besnoiti, B. tarandi* y *B. bennetti*, las especies de *Besnoitia* que afectan a ungulados no poseen cuerpos enigmáticos, estructura que si está presente en otras especies como *B. akodoni, B. jellisoni B. darlingi, B. neotomofelis* y *B. oryctofelisi.* El origen y función de los mismos todavía se desconoce (Dubey et al., 2003a, 2004, 2005).

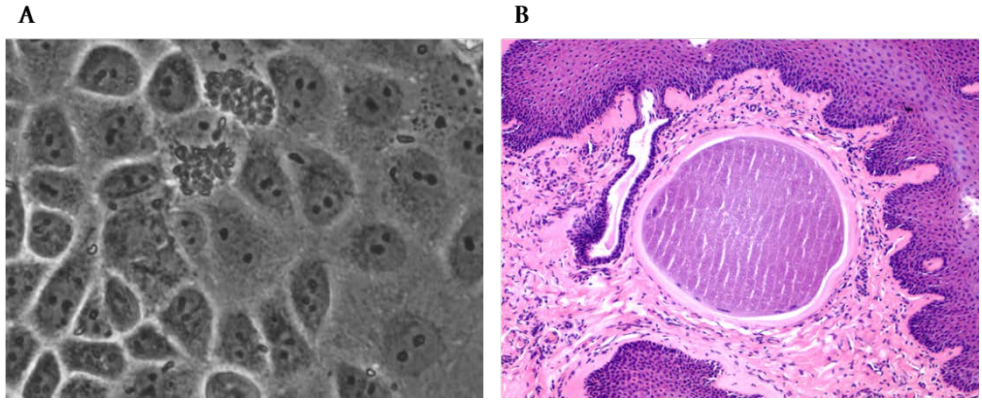
Figura 2. Ultraestructura del quiste tisular de *B. besnoiti* (imagen obtenida con TEM).



Por el momento, se desconoce el ciclo biológico completo de *B. besnoiti* (Diesing et al., 1988), pero se piensa que es heteroxeno y que tanto los bóvidos (Pols, 1960) como los antílopes (Basson et al., 1965) actúan como hospedadores intermediarios. En ellos se desarrollan los taquizoítos y los bradizoítos, que producen los signos clínicos asociados a las fases aguda y crónica de la enfermedad, respectivamente (Figuras 3 y 4). Los taquizoítos son el estadio que se multiplica rápidamente en las células endoteliales de los vasos sanguíneos. Por otra parte, los bradizoítos se agrupan y multiplican lentamente en el interior de quistes tisulares, algunos de ellos macroscópicos, que se localizan principalmente en el tejido conjuntivo subcutáneo. Ambos estadios son infectantes para los hospedadores intermediarios, como se ha demostrado en diversas infecciones experimentales (Pols 1960; Bigalke et al., 1967; Bigalke, 1968; Diesing et al., 1988).

Figura 3. Estadios de *B. besnoiti* que se desarrollan en el hospedador intermediario.

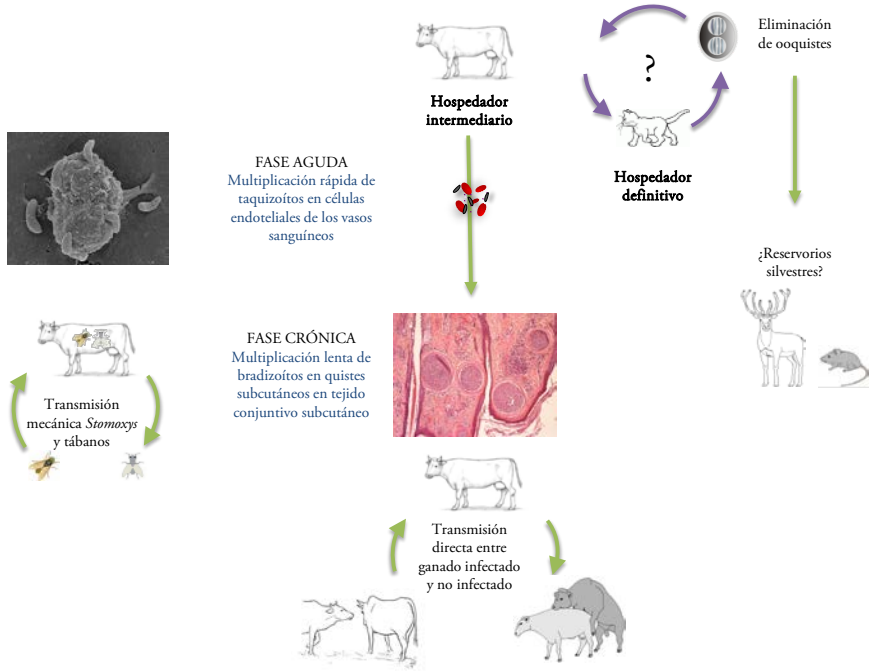
(A): taquizoítos de *B. besnoiti* en cultivo celular; (B): bradizoítos de *B. besnoiti* en el interior de un quiste tisular (H-E).



La identidad del hospedador definitivo de *B. besnoiti* no se conoce, aunque se especula con la posibilidad de que sea el gato (*Felis silvestris catus*) o algún carnívoro silvestre, como sucede en otras especies de *Besnoitia* (*B. darlingi, B. neotomofelis, B. oryctofelisi* y *B. wallacei*) (Basso et al., 2011) (Tabla 1). En este sentido, Peteshev et al. (1974) (citado por Bigalke, 1981) sugirió que tanto los gatos domésticos como silvestres (*Felis lybica)* eliminaban ooquistes de *B. besnoiti* en las heces tras la ingestión de tejidos con quistes procedentes de una vaca infectada naturalmente. Desafortunadamente, en esta época no había técnicas moleculares disponibles, por lo que no fue posible demostrar la presencia de ADN de *B. besnoiti* en las heces de los animales infectados. Rommel (1975) también observó que algunos perros y gatos eliminaban ooquistes con las heces tras la ingestión de tejidos con abundantes quistes tisulares de *B. besnoiti*. Sin embargo, éstos tampoco fueron caracterizados y no se pudo determinar su identidad. Por otra parte, Diesing et al. (1988) infectaron cuatro gatos domésticos (*Felis domestica*) y cuatro salvajes (*Caracal caracal* y *Felis chaus*), así como otros felidae silvestres (*Acitonyx jubatus, Panthera leo* y *Panthera pardus*), cánidos domésticos (*Canis familiaris*) y silvestres (*Canis mesomelas* y *Vulpes chama*), mangostas (*Mungus mungo* y *Herpestes pallodinosus)*, ginetas (*Genette genette*), una especie de buitre (*Gyps africanus*) y 20 serpientes de 11 especies diferentes, con tejidos

con quistes de *B. besnoiti* procedentes de un toro de Israel y dos vacas de Sudáfrica. Desafortunadamente, ninguno de los animales infectados eliminó ooquistes de *Besnoitia* spp. en las heces durante un periodo de al menos tres semanas. Sin embargo, se pudo observar que algunos animales infectados eliminaban ooquistes de *Isospora* spp. Por otra parte, en un estudio reciente en el que se realizaron infecciones experimentales en perros y en gatos, los resultados serológicos demostraron que *B. besnoiti* podría infectar al gato, ya que se pudo demostrar la seroconversión de los animales infectados, cinco semanas después de la ingestión de tejidos bovinos con quistes. Desafortunadamente, no fue posible demostrar la eliminación de ooquistes en las heces de ninguno de los animales infectados (Basso et al., 2011). El trabajo realizado por Marcén et al. (2011) también demostró la seroconversión de gatos tras la ingestión de tejidos con quistes de *B. besnoiti.* Además se observó la presencia de formas parasitarias en las heces de dos de los animales infectados. Sin embargo, los resultados moleculares obtenidos en este estudio no pudieron demostrar el papel de los gatos como hospedadores definitivos de *B. besnoiti*.

Figura 4. Ciclo biológico y transmisión de *B. besnoiti* (Fuente: Álvarez-García et al., 2014c).



Finalmente, los rumiantes silvestres que conviven con el ganado bovino en extensivo, deben contemplarse como posibles reservorios de la enfermedad. De hecho, Gutiérrez-Expósito et al. (2013), ha demostrado la presencia de anticuerpos específicos anti-*Besnoitia* spp. en un ciervo y en un corzo procedentes de los Pirineos (área donde la enfermedad es endémica).

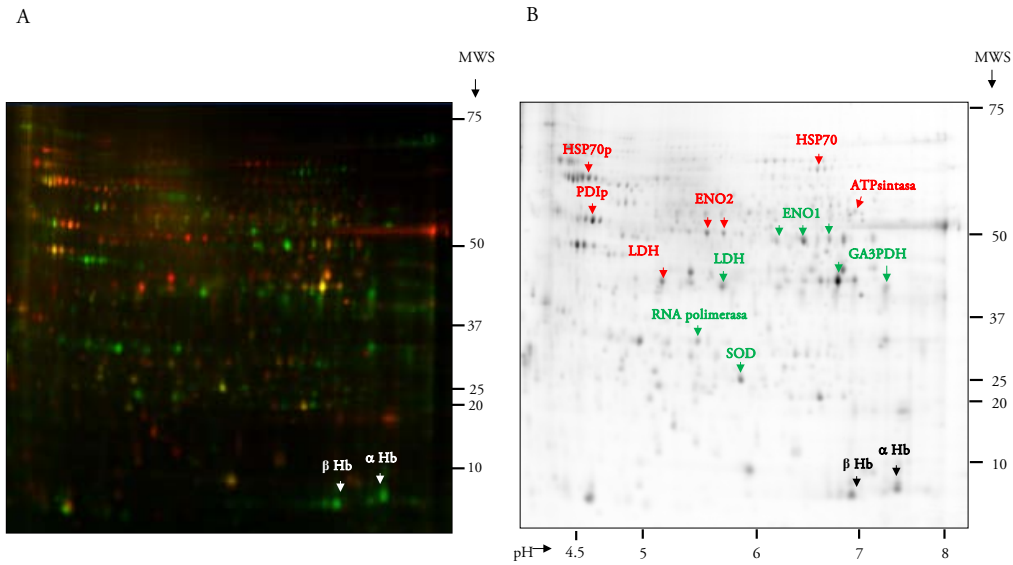
2.3. Genoma y proteoma

Desafortunadamente, hoy por hoy se desconoce la secuencia del genoma de *B. besnoiti* (revisado por Cortes et al., 2014), aunque se piensa que pueda tener un tamaño entre los 60 y 130 Mb, similar a *T. gondii, N. caninum* y *Sarcocystis* spp. (www.genedb.org; www.ncbi.nlm.nih.gov/pubmed). Tampoco se conoce su composición proteica y, por tanto, aquellas proteínas implicadas en el ciclo lítico de los taquizoítos, así como las implicadas en la conversión del taquizoíto a bradizoíto. De hecho, en la actualidad tan sólo se ha identificado y caracterizado una proteína de *B. besnoiti*, la BbPDI (Marcelino et al., 2011), la cual se piensa que puede estar secretada por los micronemas y que puede tener un papel esencial durante la invasión de la célula hospedadora, extrapolando los resultados obtenidos en *N. caninum* (Naguleswaran et al., 2005). Por otra parte, Fernández-García et al. (2013), realizó el primer estudio proteómico de *B. besnoiti* y estudió las diferencias en la composición proteica entre el estadio de taquizoíto y de bradizoíto empleando electroforesis diferencial en gel (DIGE) (Figura 5). Mediante MALDI-TOF/MS, y utilizando las secuencias disponibles de *N. caninum* y *T. gondii,* logró identificar cinco proteínas más abundantes en el estadio de bradizoíto (GA3PDH, ENO1, LDH, superóxido dismutasa (SOD) y RNA polimerasa) frente a cinco más abundantes en el estadio de taquizoíto (ENO2, LDH, HSP70, PDI y ATP sintasa). Desafortunadamente, todas ellas son enzimas presentes no solo en protozoos del filo Apicomplexa, sino también en todos los organismos eucariotas. Finalmente, Dubey et al. (2013), mediante inmunohistoquímica (IHQ) y empleando un suero policlonal desarrollado frente al antígeno específico de bradizoíto BAG-1 de *T.*

gondii, ha demostrado recientemente la presencia de esta proteína en el estadio de bradizoíto de *B. besnoiti.*

Figura 5. Geles DIGE y proteínas correspondientes a los estadios de taquizoíto y bradizoíto de *B. besnoiti* (Fuente: Fernández-García et al., 2013).

(A): gel DIGE con extractos de bradizoíto y de taquizoíto marcados con Cy3 y Cy5, respectivamente; (B): gel DIGE correspondiente al estándar interno y teñido con plata. Las proteínas identificadas mediante MALDI-TOF/MS y más abundantes en el estadio de taquizoíto están señaladas con flechas en rojo y las más abundantes en el estadio de bradizoíto en verde. Las proteínas de α y β hemoglobina identificadas están señaladas como αHb y βHb, respectivamente.



3. Besnoitiosis bovina

La besnoitiosis bovina, es una enfermedad de curso crónico y debilitante que ocasiona importantes pérdidas económicas, ya que afecta negativamente a los parámetros productivos y reproductivos. Ocasionalmente, puede producir la muerte de los animales infectados, aunque en términos generales presenta tasas de mortalidad menores del 10% (EFSA, 2010).

3.1. Distribución geográfica

La besnoitiosis bovina se ha descrito en África (Hofmeyr, 1945), donde se encuentra ampliamente distribuida en Angola, Botswana, Camerún, Congo, Kenia, Namibia, Nigeria, República de Zimbabue, Sudáfrica, Sudán, Swazilandia, Tanzania y Uganda (Chatikobo et al., 2013). Además, se ha descrito en algunas regiones de Asia (China, Corea, Israel, Kazajistán, Rusia y Uzbekistán) (revisado por Olias et al., 2011) y en Venezuela (Vogelsang y Gallo, 1941) (Figura 6).

En Europa, durante años la besnoitiosis bovina ha estado restringida a los Pirineos y a la zona del Alentejo de Portugal (zonas donde la enfermedad es tradicionalmente endémica) (Besnoit y Robin, 1912; Franco y Borges, 1916; Leitão, 1963; Bourdeau et al., 2004). Sin embargo, en los últimos 20 años, la enfermedad se ha diseminado a lo largo de la región portuguesa del Alentejo (Cortes et al., 2004; 2005; 2006b, c), y en la zona centro, este y oeste de Francia (Alzieu, 2007; revisado por Jacquiet et al., 2010). Además, en España, la Sierra de Urbasa y Andía (Navarra) y la zona del Maestrazgo (Teruel) también se consideran como zonas donde la besnoitiosis bovina es endémica (revisado por Álvarez-García et al., 2013). Igualmente, en 1994 se detectaron animales infectados entre el ganado exportado a Italia procedente de Francia (Agosti et al., 1994), y, desde 2009, se han descrito brotes autóctonos de la enfermedad en la zona norte de los Montes Apeninos, donde ya se considera que la besnoitiosis bovina es endémica (Gentile et al., 2012).

Por otra parte, durante los últimos años se han descrito numerosos brotes en el norte y en el centro de España (Fernández-García et al., 2010; revisado por Álvarez-García et al., 2013), así como en la frontera con Portugal (EFSA, 2010), los cuales se asociaron, inicialmente, a la compra de animales infectados procedentes de zonas donde la enfermedad era endémica. Del mismo modo, una granja de ganado de carne de la zona alemana de Bavaria, se vio afectada debido probablemente a la importación de bovinos de las razas Charolesa y Limusina desde Francia (Majzoub et al., 2010; Rostaher et al., 2010). Tras esta diseminación de la enfermedad desde Francia hacia Italia y Alemania, principalmente asociada al comercio con animales infectados, en Suiza, Lesser et al.

(2012), analizaron el ganado bovino importado desde otras zonas donde la enfermedad ya había sido descrita y detectaron que dos animales procedentes de Francia y dos procedentes de Alemania, estaban infectados. Posteriormente, en 2013, se detectaron los primeros casos de besnoitiosis bovina en ganado autóctono (Basso et al., 2013). Del mismo modo, en Croacia (Beck et al., 2013) se ha observado, recientemente, que el 50% de los animales importados desde Francia, están infectados. La última descripción de la enfermedad en Europa procede de Hungría (Hornok et al., 2014), donde, tras importar 178 animales aparentemente sanos procedentes de Francia, se han descrito los primeros casos clínicos de besnoitiosis bovina, tanto en el ganado importado como en ganado autóctono (Figura 6). Además, en otros países mediterráneos como Grecia, se sospecha de la presencia de la enfermedad, ya que se han detectado animales seropositivos en explotaciones lecheras, que albergaban animales procedentes de Alemania, Francia y los Países Bajos. Sin embargo, no se han registrado animales con signos clínicos y la presencia de la enfermedad en este país debería ser confirmada (Papadopoulos et al., 2014).

Figura 6. Distribución geográfica de la besnoitiosis bovina.

En estos estudios se ha puesto de manifiesto que la aparición de nuevos brotes de la enfermedad está asociada, fundamentalmente, a la circulación de ganado bovino sin estatus sanitario conocido. Este movimiento de animales, se ha visto favorecido por el incremento del censo de ganado bovino de carne en Europa. Sin embargo, cabe destacar que, la incorporación de animales infectados, las prácticas de manejo asociadas al ganado de carne (como son el uso de pastos comunales, la monta natural y la trashumancia) y el incremento de poblaciones de vectores artrópodos debidas al cambio climático pueden haber favorecido la transmisión horizontal del parásito y, por tanto, la diseminación de la enfermedad y la aparición de casos autóctonos (revisado por Álvarez-García et al., 2013). Por ello, debido al aumento del número de casos notificados y a la expansión geográfica, la besnoitiosis bovina está considerada actualmente como una enfermedad re-emergente en Europa, lo cual pone de manifiesto el riesgo que supone esta enfermedad para otros países vecinos (revisado por Álvarez-García et al., 2013).

3.2. Prevalencia e incidencia

Inicialmente, los primeros estudios de prevalencia llevados a cabo en Sudáfrica describieron bajas prevalencias de entre el 10% y el 50%, probablemente debido a que el diagnóstico tan solo se basaba en la detección de quistes en la conjuntiva ocular y de lesiones en la piel (Bigalke, 1968). Sin embargo, los primeros estudios serológicos que se llevaron a cabo en el Sur de África y en Israel, ya describieron tasas de prevalencia de entre el 64,4 y el 66,9% en ganado de carne de Israel (Neuman, 1972; Frank et al., 1977) y del 50% en animales sin signos clínicos procedentes de Sudáfrica (Janitschke et al., 1984).

Sin embargo, los estudios de prevalencia de la infección en Europa, todavía son escasos. En Francia se ha observado una tasa de seroprevalencia intra-rebaño del 50% en una explotación de leche localizada en una zona libre de la enfermedad (Genest, 2008). En España, dos estudios serológicos recientes han puesto de manifiesto la importancia de esta enfermedad. En un estudio transversal realizado en ganado bovino de carne de Navarra, se ha descrito una tasa de seroprevalencia individual del 16%. Además, los animales seropositivos estaban localizados en zonas de montaña donde previamente se

habían detectado casos clínicos de la enfermedad (Sierra de Urbasa y Andía y los Pirineos) (Álvarez-García et al., 2014a). Posteriormente, se realizó el primer estudio de prevalencia individual en machos y el primer estudio de prevalencia de rebaño en una zona de Europa donde la enfermedad es endémica (Pirineo oscense). Tras analizar un total de 3.211 hembras de 63 rebaños y todos los sementales de esta zona (587) se estimó una prevalencia del 48,7% en machos y del 51,9% en hembras, y una prevalencia de rebaño del 87,3%, indicando que la enfermedad se encuentra ampliamente distribuida (Gutiérrez-Expósito et al., 2014). Por otra parte, la besnoitiosis bovina se detectó por primera vez en una explotación localizada en el noroeste de Italia en ganado de carne procedente de Francia (Agosti et al., 1994). Posteriormente, Gentile et al. (2012) realizó el seguimiento de esta misma explotación y describió los primeros casos autóctonos de la enfermedad, notificando una seroprevalencia del 41,2% con un 23,4% de los animales con signos clínicos, lo que demuestra la existencia de zonas endémicas de la enfermedad en Italia. Sin embargo, un estudio reciente ha descrito una baja prevalencia de la enfermedad (0,3% y 3,9% de seroprevalencia individual e intra-rebaño, respectivamente) en la zona noroeste (Lombardia, Piemonte y Liguria) y en la isla de Cerdeña (Gazzonis et al., 2014). Recientemente, Waap et al. (2014) en un estudio transversal estratificado llevado a cabo en Portugal, han descrito una seroprevalencia del 5,1% y una seroprevalencia media intra-rebaño del 33%, con la mayoría de los casos localizados en la región del Alentejo.

Sin embargo, la información sobre la prevalencia de signos clínicos, hoy por hoy es muy escasa, y todos los datos disponibles proceden de brotes de la enfermedad tanto en zonas donde la besnoitiosis bovina es endémica, como en zonas libres de la enfermedad con anterioridad. Por ello, en estos estudios sólo se describen datos de prevalencia intra-rebaño. Además, los trabajos realizados hasta el momento son poco comparables, fundamentalmente debido al diferente tamaño muestral, así como las diferentes técnicas serológicas utilizadas en cada uno de los estudios. Por una parte, en explotaciones localizadas en zonas donde la enfermedad es endémica, como el estudio realizado por Cortes et al. (2005) en la zona Sur de Portugal, se ha descrito que el 42% de los machos aparentemente sanos muestreados, presentaba quistes de *B. besnoiti* que fueron visibles

mediante histopatología. Además, en el seguimiento de una explotación localizada en la zona del Alentejo, donde previamente se había descrito la presencia de la enfermedad, se observó un aumento de la seroprevalencia del 36% al 70% en un periodo de 18 meses (Cortes et al., 2006b). Igualmente, en un estudio llevado a cabo en una explotación localizada en el noroeste de Francia, zona donde la enfermedad también es endémica, se describió una seroprevalencia individual del 33,8%, con un 10% de los animales con signos clínicos. El seguimiento de la misma explotación durante 14 meses, reveló un aumento de la seroprevalencia (89,5%) y un mantenimiento del porcentaje de animales con signos clínicos (12,3%) (Liénard et al., 2011). Por otra parte, en zonas no endémicas y donde aparición de la enfermedad es reciente, como el brote descrito por Fernández-García et al. (2010) en la Sierra de Guadalajara, el 90,8% de las hembras y el 71,4% de los machos fueron seropositivos. Además el 43,2% de los animales presentaron algún signo clínico. En brotes más recientes, como el descrito en Croacia, se ha observado que el 42% de los animales de una explotación estaban infectados, mientras que el 37% mostraba signos clínicos (Beck et al., 2013). Finalmente, en una explotación localizada en el este de Hungría, se observó que 12 animales estaban infectados y que 10 de ellos mostraban signos clínicos de besnoitiosis bovina. Desafortunadamente, en este estudio el resto del rebaño no fue muestreado (Hornok et al., 2014). Finalmente, en relación a los brotes recientemente notificados en Egipto y Grecia, las tasas de seroprevalencia individual del 13,5% y del 22%, respectivamente, descritas usando ELISAs (Ashmawy y Abu-Akkada, 2014; Papadopoulos et al., 2014), así como las tasas de seroprevalencia individual y de rebaño del 6% y del 28,7%, respectivamente, observadas en Jordania (Talafha et al., 2015), deberían ser confirmadas mediante otras técnicas serológicas, ya que ninguno de los animales muestreados presentó signos clínicos compatibles con la enfermedad.

Como podemos observar, la seroprevalencia en las zonas afectadas oscila entre el 15% y el 95% (Gutiérrez-Expósito et al., 2014). Además, estos valores se mantienen elevados incluso cuando la enfermedad se establece de forma endémica en una zona y los casos

clínicos disminuyen (García-Lunar et al., 2010; Liénard et al., 2011; Gutiérrez-Expósito et al., 2015).

En relación a la incidencia de la enfermedad, el único estudio realizado en España se ha llevado a cabo en la Sierra de Urbasa y Andía, es decir, en una zona donde la enfermedad es endémica, y ha descrito una tasa de incidencia serológica del 22%, junto con una incidencia clínica de entre el 12% y el 17% durante un periodo de 4 años, lo que pone de manifiesto la alta tasa de transmisión del parásito a lo largo del tiempo (Gutiérrez-Expósito et al., 2015).

3.3. Transmisión

La transmisión horizontal parece ser la responsable de la diseminación de la enfermedad, ya que se ha encontrado una asociación significativa entre mayores tasas de prevalencia y un incremento de la edad de los animales (Bigalke, 1981; Fernández-García et al., 2010). Es probable que ésta tenga lugar, principalmente, mediante el contacto directo entre animales infectados y no infectados a través de heridas o escoriaciones, teniendo en cuenta que se ha demostrado que el estadio de bradizoíto es capaz de atravesar las mucosas (Bigalke, 1968). De hecho, diversos autores han señalado un aumento estacional de los casos clínicos durante el verano, cuando los rebaños de ganado de aptitud cárnica comparten pastos (Alzieu, 2007; Fernández-García et al., 2010). Además, se ha demostrado el riesgo que supone introducir un animal infectado en una zona/rebaño libre de la enfermedad (revisado por Álvarez-García et al., 2013; Frey et al., 2013a, b). En el ganado de carne, dada la importancia de la monta natural, los machos utilizados como sementales son los más comercializados y la transmisión por contacto directo durante la monta, suponiendo que los quistes superficiales presentes en las membranas mucosas se puedan romper, no se puede descartar (Castillo et al., 2009; Fernández-García et al., 2010; revisado por Álvarez-García et al., 2013). Por otra parte, en el ganado bovino se ha demostrado, experimentalmente, que algunas especies de tábanos (*Atylotus nigromaculatus, Tabanocella denticornis,* and *Haematopota albihirta*) y moscas de los

establos (*Stomoxys calcitrans*) pueden intervenir en la transmisión mecánica de *B. besnoiti*. Sin embargo, parece que es necesario un número elevado de picaduras para que se produzca una transmisión eficaz. Por ello, este modo de transmisión podría también contribuir al aumento del número de casos estivales, época en la que estos vectores presentan una mayor actividad. Por otra parte, parece que el tiempo de supervivencia del parásito en los insectos es limitado, por lo que parece razonable descartar la posibilidad de diseminación a larga distancia (Bigalke, 1968). Del mismo modo, los resultados obtenidos por Gollnick et al. (2015) sugieren que la importancia de los vectores en la diseminación del parásito es escasa, ya que, incluso cuando la enfermedad se transmitió eficazmente desde animales infectados a animales sanos en un espacio cerrado, tan solo un ejemplar de *S. calcitrans* de los 48 muestreados presentó ADN de *B. besnoiti*. En este estudio, ninguno de los 111 ejemplares de *Musca domestica, M. autumnalis* y *Haemotobia irritans* que fueron capturados, presentaron ADN del parásito. Además, no se debe descartar la posible participación de los rumiantes silvestres, los roedores u otras especies como reservorios del parásito en la transmisión (Bigalke, 1981; Mehlhorn et al., 2009; Gutiérrez-Expósito et al., 2013). Por otra parte, la transmisión horizontal por la ingestión de ooquistes, todavía no se ha esclarecido. Finalmente, cabe destacar que la transmisión venérea parece improbable ya que, hoy por hoy, no se ha podido detectar ADN de *B. besnoiti* en el semen de machos naturalmente infectados (Esteban-Gil et al., 2014). Además, no se ha podido comprobar la transmisión vertical del parásito (vía transplacentaria o lactogénica). Sin embargo, el aumento de la prevalencia conforme se incrementa la edad de los animales, junto con el hecho de que madres crónicamente infectadas tienen descendencia seronegativa (Shkap et al., 1994) sugiere una escasa o nula importancia de éste modo de transmisión. En apoyo de esta hipótesis, hay que señalar que tan sólo se ha descrito, hasta el momento, un caso clínico en un animal menor de seis meses (Diezma-Díaz et al., 2015).

3.4. Factores de riesgo

La identificación de los factores de riesgo tiene un gran interés para la elección y la implementación de medidas de control eficaces frente a esta enfermedad. Sin embargo, debido a la falta de conocimiento sobre el hospedador definitivo de *B. besnoiti* o sobre sus posibles reservorios silvestres, es difícil, en muchos casos, establecer planes de control con ciertas garantías de éxito. Los factores de riesgo de la infección por *B. besnoiti* conocidos se han identificado por medio de evidencias epidemiológicas. Entre ellos, podemos destacar los factores dependientes del manejo y los factores individuales.

En cuanto a los primeros, el movimiento de animales infectados desde zonas endémicas a zonas libres supone el mayor riesgo para la diseminación de la enfermedad. Además, también influyen de forma notable aquellas prácticas asociadas al manejo de razas de aptitud cárnica, donde se practica la monta natural y en las que los animales comparten pastos, ya que puede existir mayor contacto directo entre ganado infectado y sano, así como con otros posibles reservorios silvestres. Además, se ha descrito cierta estacionalidad de la enfermedad, ya que la mayoría de los casos nuevos ocurren durante los meses cálidos y con niveles de humedad relativamente altos, cuando el ganado comparte pastos (Alzieu, 2007; Fernández-García et al., 2010; Gutiérrez-Expósito et al., 2015) y los artrópodos hematófagos, que pueden actuar como vectores del parásito, están en su periodo de máxima actividad.

En relación a los factores individuales, cabe destacar que se ha observado un incremento de la seroprevalencia conforme aumenta la edad de los animales (Bigalke, 1968; Fernández-García et al., 2010). Por ello, los animales adultos tienen más posibilidades de infectarse, ya que el tiempo de exposición es mayor que en los jóvenes. En este sentido, tan sólo se ha descrito un caso clínico de besnoitiosis bovina en una hembra menor de seis meses (Diezma-Díaz et al., 2015), si bien Hornok et al. (2014) han detectado la presencia de anticuerpos frente a *B. besnoiti* en animales con edades comprendidas entre los seis y los 12 meses. Estos hechos pueden sugerir un papel protector para los anticuerpos calostrales (Diezma-Díaz et al., 2015) así como una cierta resistencia de los animales jóvenes a la infección por *B. besnoiti*, la cual ya ha sido descrita

en infecciones por otros protozoos como *Babesia bovis* (revisado por Brown et al., 2006). No obstante, y a diferencia de la seroprevalencia, los signos clínicos de la enfermedad se suelen observar con mayor frecuencia en animales de 2 a 4 años en comparación con los mayores de 4 años. De hecho, los animales crónicamente infectados pueden experimentar una aparente disminución de la gravedad de los signos clínicos y ser resistentes a las reinfecciones, pero permanecen infectados de por vida y sus producciones quedan mermadas (Kumi-Diaka et al., 1981; Nobel et al., 1981). De esta forma, se ha observado que en zonas donde la enfermedad es endémica, un porcentaje alto de animales infectados que presentan anticuerpos específicos frente a *B. besnoiti,* no presentan signos clínicos detectables (infección subclínica) (Gutiérrez-Expósito et al., 2015).

Finalmente, cabe destacar que todas las razas son susceptibles a la infección por *B. besnoiti,* sin embargo se ha descrito principalmente en razas de aptitud cárnica, asociado a las prácticas de manejo (Álvarez-García et al., 2014a). De hecho, la descripción de la infección en explotaciones lecheras corresponde a trabajos puntuales (Goldmand y Pipano, 1983; Liénard et al., 2013; datos no publicados). Además, el sexo no se considera un factor de riesgo ya que machos y hembras presentan similares tasas de seroprevalencia. No obstante, en estudios realizados en Francia se ha observado que los toros son seropositivos con mayor frecuencia que las vacas y los signos clínicos en sementales parecen ser más graves (Fernández-García et al., 2010; revisado por Jacquiet et al., 2010; Gutiérrez-Expósito et al., 2014). En las explotaciones de leche infectadas, se ha señalado la presencia de aborto en algunas hembras infectadas, hecho que ocurre, fundamentalmente, durante la fase aguda (datos no publicados). Por último, se piensa que los fenómenos fisiológicos o patológicos que originan una inmunosupresión pueden ser responsables de que tan sólo algunos animales infectados desarrollen signos clínicos.

3.5. Patogenia, signos clínicos y lesiones

Los signos clínicos comienzan a desarrollarse tras un periodo de incubación de aproximadamente 2 semanas de duración (revisado por Álvarez-García et al., 2014c). La

enfermedad comienza con una fase aguda febril, que dura aproximadamente 12-13 días, caracterizada por la aparición de signos clínicos inespecíficos que pueden pasar desapercibidos como fiebre (40,8-41,6 ºC), taquicardia, taquipnea, linfadenitis, inapetencia y pérdida de peso (revisado por Álvarez-García et al., 2014c; Gollnick et al., 2015). En la hembra, el cuadro febril, puede ocasionar el aborto. A esta fase le sigue una fase aguda de anasarca, que puede prolongarse hasta las 4-5 semanas post infección (spi), y que se caracteriza por la presencia de edemas que se localizan fundamentalmente en la cabeza y el cuello y se desplazan hacia las partes inferiores del animal, como pecho, papada, prepucio, ubre y extremidades (Figura 7A). Estos signos clínicos se asocian con la proliferación de taquizoítos en las células endoteliales de los vasos sanguíneos produciéndose un importante fallo circulatorio caracterizado por vasculitis, trombosis y necrosis de vénulas y arteriolas. Además puede aparecer hiperemia conjuntival, lacrimeo, fotofobia y descarga ocular y nasal mucopurulenta (Figura 7B), y en los machos es frecuente la aparición de orquitis necrotizante (Figura 7C). En los casos graves pueden aparecer edemas alveolares e intersticiales en pulmones con los consiguientes signos respiratorios y también se ha descrito la aparición de edema articular, aunque estudios recientes han mostrado que es poco frecuente en condiciones naturales (Gollnick et al., 2015) (Figura 7D). Durante esta fase comienzan a desarrollarse numerosos quistes tisulares en la capa íntima de los vasos sanguíneos con especial tropismo por el tejido conjuntivo.

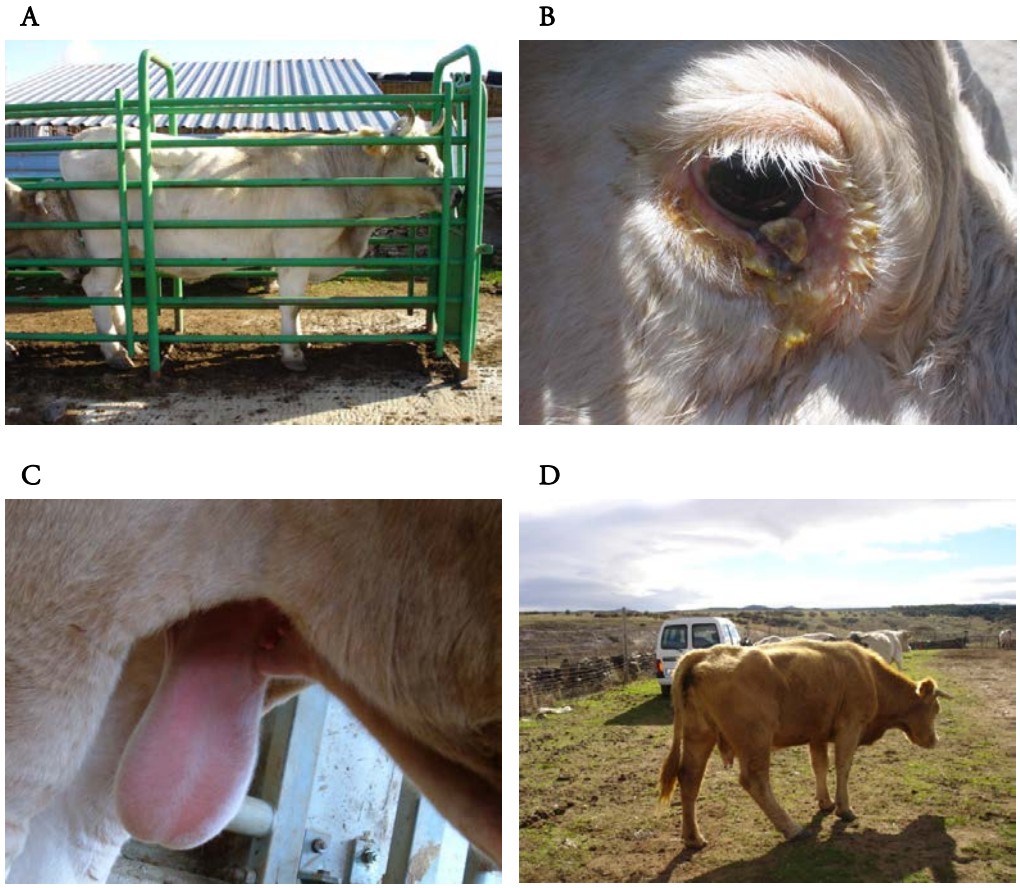
Durante la fase crónica, conocida como fase de escleroderma, se produce un deterioro gradual de la condición corporal, pérdida de peso y, ocasionalmente, mortalidad. Además, a partir de las 6-7 spi, se pueden observar quistes macroscópicos que se visualizan inicialmente en las conjuntivas palpebral y esclerótica y, posteriormente, en el vestíbulo vaginal (Figura 7E y F), los cuales muchas veces constituyen el único signo de enfermedad (Pols, 1960; Bigalke, 1968, 1981; Fernández-García et al., 2010; Rostaher et al., 2010; Gollnick et al., 2015). Éstos también se desarrollan en el endotelio, dermis, fascias, mucosa del tracto respiratorio superior, faringe, y en los machos, en el testículo y en el epidídimo, entre otras localizaciones. Curiosamente, se ha detectado, recientemente,

ADN parasitario en el SNC de animales crónicamente infectados (Basso et al., 2013), si bien queda por esclarecer si *B. besnoiti* es capaz de formar quistes en esta localización. Como consecuencia del desarrollo de quistes en el tejido subcutáneo, se puede observar engrosamiento, endurecimiento y formación de pliegues en la piel ("piel de elefante") (Bigalke, 1981) (Figura 7G y H). En las zonas de mayor carga parasitaria se puede observar alopecia e incluso desprendimiento de fragmentos necrosados de la epidermis. En los machos, la presencia de quistes tisulares en diferentes localizaciones del aparato genital, puede producir infertilidad (Neuman, 1962; Kumi-Diaka et al., 1981; Dubey et al., 2013). En particular, los quistes tisulares parecen tener tropismo por las paredes vasculares del plexo pampiniforme, lo que produce una disminución del aporte sanguíneo a los testículos (Kumi-Diaka et al., 1981). Además, los quistes localizados entre los túbulos seminíferos, ejercen una presión directa sobre el tejido, produciendo necrosis y mineralización y, posteriormente, atrofia testicular y epididimal (Kumi-Diaka et al., 1981; Dubey et al., 2013). Estos acontecimientos, junto con el pobre intercambio de calor que se produce como consecuencia del engrosamiento de la piel del escroto, son responsables de la aparición de azoospermia e infertilidad (Figura 7I y 8) (Bigalke, 1968; Kumi-Diaka et al., 1981; Bigalke y Prozesky, 1994). En las hembras, también pueden aparecer grietas y nódulos visibles en los pezones y esta situación se puede complicar con infecciones secundarias oportunistas (Figura 7J). Además, se ha observado que los animales infectados pueden desarrollar laminitis crónica y presentar úlceras en las pezuñas, lo que puede llegar a producir postración (Gollnick et al., 2015). Estos signos se habían relacionado tradicionalmente con los edemas articulares que pueden observarse durante la fase aguda de la enfermedad (Bigalke y Prozesky, 2004; revisado por Jacquiet et al., 2010). Sin embargo, diversos estudios han observado, recientemente, que la aparición de laminitis está asociada a la fase crónica (Gollnick et al., 2015) (Figura 7D). En este sentido, se ha sugerido que la presencia de quistes en el corion laminar junto con los que se desarrollan en los vasos de la dermis, favorecen el desarrollo de laminitis, propiciándose una desigual distribución del peso, que a su vez puede resultar en la

formación de úlceras soleares y la postración de los animales afectados (Gollnick et al., 2015).

Figura 7. Signos clínicos asociados a la infección por *B. besnoiti* durante las fases aguda (A-D) y crónica (E-I).

(A): edema en papada y pecho; (B): secreción ocular mucopurulenta; (C): orquitis; (D): cojera; quistes macroscópicos en (E): conjuntiva ocular y (F): mucosa vulvar; (G, H): engrosamiento, endurecimiento, formación de pliegues en la piel con zonas de alopecia y desprendimiento de la piel necrótica. Se puede observar (G): secreción ocular mucopurulenta; (I): degeneración testicular y (J): nódulos en los pezones.



E

F

G

H

I

J

En relación a las alteraciones hematológicas y bioquímicas que se producen como consecuencia de la infección, éstas han sido recientemente estudiadas por Langenmayer et al. (2015c). Aunque no se han descrito alteraciones notables, se ha podido detectar que

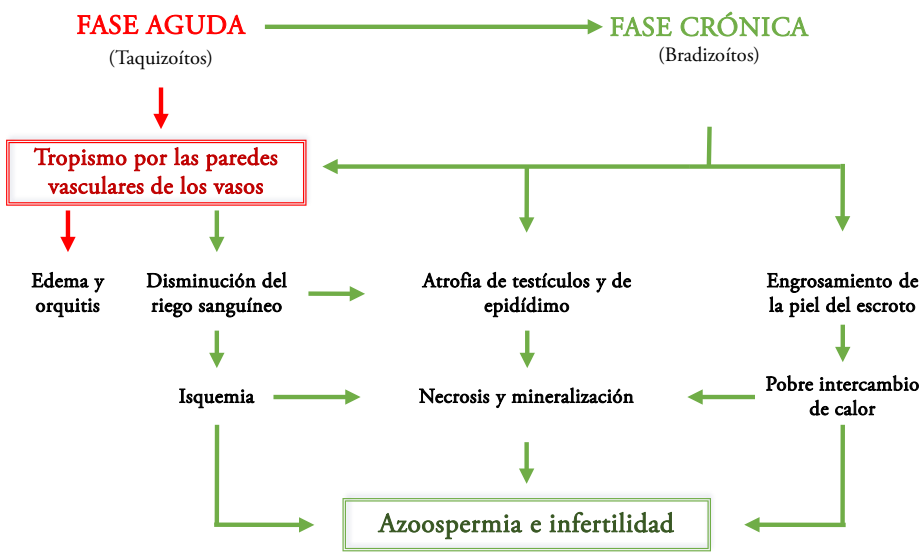
los animales infectados pueden presentar leucopenia y anemia, así como una disminución de la concentración de calcio y de la albúmina sérica bovina. Sin embargo, dado que ninguno de los animales estudiados mostraba signos clínicos compatibles con hipoalbuminemia, se ha sugerido que tanto la inflamación como el balance energético negativo, que se produce por el aumento de la demanda de energía debido a la formación de múltiples quistes tisulares parasitarios, puedan ser los responsables de esta alteración. Además, se ha observado una disminución de los niveles de urea y magnesio, que se han asociado a la anorexia durante los primeros días de infección. Por otra parte, aunque el aumento de la creatinina se ha asociado a la deshidratación, también puede deberse a una degeneración muscular que puede producirse en los animales gravemente afectados. Así mismo, se ha subrayado la importancia de la pérdida de la integridad de las fibras musculares durante la fase aguda, lo que puede ser responsable del aumento de la actividad enzimática de la aspartato transaminasa y de la creatinin-quinasa. Por último, tanto durante la fase aguda como en la fase crónica, se pueden registrar concentraciones altas de globulinas, que se atribuyen, principalmente, al desarrollo de anticuerpos específicos frente al parásito.

Cabe destacar que, aunque algunos animales infectados pueden morir durante las fases aguda y/o crónica, la besnoitiosis bovina presenta tasas de mortalidad muy bajas, menores del 10%. (Pols, 1960; Schulz, 1960; EFSA, 2010). En un rebaño infectado, el número de animales que muestran signos de enfermedad aguda o crónica representan tan solo la punta del iceberg tanto en zonas donde la enfermedad es endémica (1-10%) como en brotes epidémicos (15-40%) (Legrand, 2003; revisado por Jacquiet et al., 2010; Fernández-García et al., 2010; Liénard et al., 2011). Además, un pequeño porcentaje de animales con baja carga parasitaria tan solo presentan quistes macroscópicos en la conjuntiva ocular y/o en la región vulvar, los cuales muchas veces son difíciles de detectar. Estos animales representan porcentajes muy bajos en brotes de la enfermedad (1-3%) (Fernández-García et al., 2010; Schares et al., 2010), aunque pueden representar entre el 15% y el 23% en zonas donde la enfermedad es endémica (Liénard et al., 2011; Gutiérrez-Expósito et al., 2015). Sin embargo, el porcentaje más alto (hasta un 17% y un 40% en

zonas donde la enfermedad es endémica y en brotes, respectivamente) está representado por animales infectados con baja carga parasitaria (infección subclínica), que representan un factor de riesgo muy importante para la transmisión del parásito (Fernández-García et al., 2010; Liénard et al., 2011; Basso et al., 2013; Frey et al., 2013a, b; Gutiérrez-Expósito et al., 2015).

Finalmente, la enfermedad es responsable de importantes pérdidas económicas asociadas a la pérdida de peso, la disminución de la producción de leche, la depreciación del valor del cuero, la esterilidad en los machos y los abortos en las hembras. Desafortunadamente, el impacto económico de la besnoitiosis bovina en Europa todavía no se conoce con exactitud.

Figura 8. Patogenia de la infección por *B. besnoiti* en el aparato genital del macho (Fuente: Álvarez-García et al., 2014c).



3.6. Respuesta inmunitaria

Besnoitia besnoiti es un parásito intracelular obligado que induce en el hospedador una respuesta inmunitaria tanto celular, como humoral. Sin embargo, se desconocen muchos factores asociados a estas respuestas. Por el momento, en relación a la respuesta innata, tan sólo algunos estudios *in vitro* han demostrado que tanto los neutrófilos como los monocitos bovinos son capaces de inducir la formación de trampas extracelulares como mecanismo de defensa frente a los taquizoítos (Muñoz-Caro et al., 2014a, b). Por otra parte, múltiples estudios han demostrado la importancia de la respuesta inmunitaria celular, predominantemente Th1, para el control de la infección de otros parásitos intracelulares taxonómicamente cercanos (*T. gondii* y *N. caninum*) y, por ello, se cree que pueda tener la misma importancia en la protección frente a infecciones por *B. besnoiti*. De hecho, se ha demostrado que los ratones knockout para INF-γ son sensibles a la infección por diversas especies de *Besnoitia* como *B. besnoiti, B. bennetti, B. tarandi, B. neotomofelis* y *B. oryctofelisi* (Dubey et al., 2003a, 2004, 2005; Schares et al., 2009; Dubey y Yabsley, 2010). Sin embargo, el motivo por el cual algunos animales adquieren la infección y desarrollan signos clínicos mientras que otros no la adquieren, se desconoce, si bien es cierto que una bajada en la capacidad de respuesta podría ser responsable de este hecho.

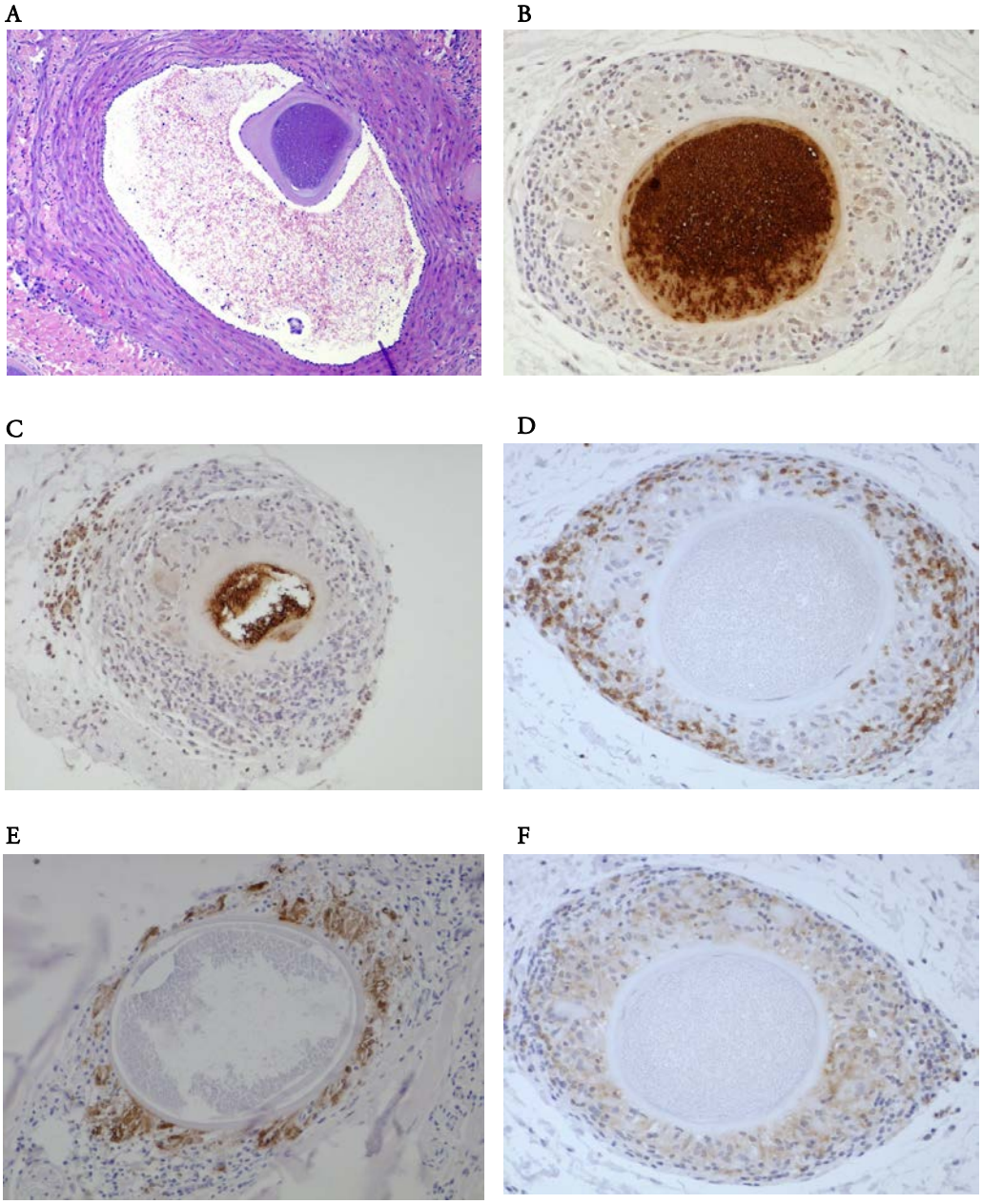
Tampoco se conoce el papel de la respuesta inmunitaria humoral durante la infección por *B. besnoiti*, aunque sí se ha demostrado que los animales infectados desarrollan, en su mayoría, anticuerpos específicos frente al parásito aproximadamente a las 2-3 spi. Además, los anticuerpos pueden detectarse durante largos periodos de tiempo, lo que facilita el diagnóstico de los animales infectados por serología. Por otra parte, parece que los animales con quistes, presentan mayores niveles de anticuerpos frente a *B. besnoiti* que los animales sin quistes visibles (Liénard et al., 2011; Schares et al., 2013). En este sentido, Gollnick et al. (2015) ha observado que en los animales infectados, la concentración de globulinas totales aumenta progresivamente desde el día de la seroconversión en adelante, a medida que se desarrollan anticuerpos específicos frente al parásito. Por otra parte, el papel protector de los anticuerpos calostrales todavía se desconoce. Sin embargo, hoy por

hoy, el hecho más sorprendente es que una vez que un animal se infecta, adquiere inmunidad frente a reinfecciones, sentándose así las bases para el desarrollo de estudios vacunales.

En relación a la respuesta inmunitaria que se produce en el entorno de los quistes tisulares, cabe destacar que se han detectado tanto quistes maduros sin reacción inflamatoria periférica, como otros rodeados por una respuesta celular de linfocitos T y B y macrófagos activados (Figura 9). Se ha descrito también la presencia de quistes rotos o degenerados que dan lugar a una reacción granulomatosa (Schulz, 1960; Nobel et al., 1981; Frey et al., 2013a, b). Curiosamente, también se ha observado que los animales con quistes rotos, presentan mayores niveles de anticuerpos, lo cual se ha atribuido a una mayor exposición del parásito al sistema inmunitario del hospedador (Frey et al., 2013a, b). También se ha sugerido que una posible reactivación del parásito sería la responsable del aumento del nivel de anticuerpos, como consecuencia de una re-exposición de los antígenos parasitarios al sistema inmunitario del hospedador. Sin embargo, en la actualidad no existen evidencias que demuestren la existencia de reactivación parasitaria, ni se han descrito mecanismos de conversión del bradizoíto a taquizoíto (Gutiérrrez-Expósito et al., 2015).

Figura 9. Quistes tisulares de *B. besnoiti* procedentes de biopsias de animales subclínicamente infectados (Fuente: Frey et al., 2013a, b).

(A): quiste tisular de *B. besnoiti* en el endotelio (H-E); (B): IHQ: quiste tisular en faringe. Nótese el infiltrado inflamatorio alrededor del quiste; (C): quiste tisular roto de *B. besnoiti;* nótese el marcado de antígenos fuera de la estructura del quiste; (D): detección de receptores CD79 (linfocitos B) en el infiltrado inflamatorio que rodea un quiste tisular; (E): detección de receptores CD3 (linfocitos T) en el infiltrado inflamatorio que rodea un quiste tisular; (F): detección de macrófagos activados (anticuerpo MAC387).



3.7. Diagnóstico

En la actualidad existe una amplia batería de técnicas disponibles para el diagnóstico de la infección por *B. besnoiti* en el ganado bovino, pero no todas aportan la misma información o tienen la misma fiabilidad. En general, el protocolo de diagnóstico debe incluir la combinación de una inspección clínica y un diagnóstico serológico, con el fin de detectar tanto animales con signos clínicos seronegativos, como animales infectados sin signos clínicos detectables. Estos últimos pueden representar la mayoría en un rebaño infectado.

3.7.1. Diagnóstico epidemiológico

La información proporcionada por el propietario en relación al manejo de la explotación es esencial para realizar un diagnóstico epidemiológico correcto de la besnoitiosis bovina. En primer lugar, es importante determinar si la explotación infectada se encuentra en una zona donde la enfermedad es endémica, o por el contrario se trata de una zona libre de besnoitiosis bovina. En función de este factor, deberemos tener en cuenta que, aquellas prácticas de manejo que habitualmente se realizan en explotaciones de vaca nodriza, como son el uso de pastos comunales y el empleo de monta natural, favorecen la transmisión de la enfermedad, sobre todo si se trata de zonas donde la enfermedad es endémica. En estos casos, hay que tener en cuenta que los signos clínicos aparecen, frecuentemente, tras la época estival, en la cual animales de diferentes rebaños comparten pastos, y están expuestos a vectores artrópodos y potenciales reservorios silvestres. Si por el contrario se trata de una zona libre de la enfermedad, deberemos prestar especial atención a las nuevas entradas en la explotación, ya que la aparición de nuevos casos está, generalmente, asociada a la incorporación de animales infectados con signos clínicos que inicialmente pasaron desapercibidos.

3.7.2. Diagnóstico clínico y lesional

Durante las primeras semanas de la infección, el diagnóstico es difícil debido a la presencia de signos clínicos inespecíficos como hipertermia, depresión, taquicardia, taquipnea, anorexia y pérdida de peso, que se pueden confundir con otras enfermedades o incluso pueden ser leves y pasar desapercibidos. A medida que la infección progresa se producen edemas marcados en zonas declives como extremidades, papada, pecho y escroto, los cuales pueden ser más fáciles de detectar. Por el contrario, es durante la fase crónica cuando aparecen las lesiones características, que son útiles para detectar los animales infectados y que ya han sido descritas en la sección 3.5. de la presente Tesis Doctoral (Figura 7E-J). Estas consisten en un progresivo endurecimiento e hiperqueratosis que se acompañan de la formación de pliegues en la piel y con la aparición de zonas alopécicas. En las hembras es frecuente encontrar nódulos y grietas en pezones y en los machos se puede llegar a visualizar una degeneración testicular. Por el contrario, la presencia de quistes tisulares en la conjuntiva ocular y en la mucosa vulvar pueden ser difíciles de detectar mediante inspección visual, sobre todo cuando se presentan en un número bajo.

3.7.3. Diagnóstico diferencial

Por el cuadro febril y los signos clínicos inespecíficos que aparecen durante la fase aguda de la infección, en el diagnóstico diferencial deben contemplarse otras enfermedades de gran importancia veterinaria como la Lengua Azul o la Fiebre Catarral Maligna. Por otra parte, cualquier enfermedad que curse con alteraciones cutáneas, como sarna, dermatofitosis, fotodermatitis o incluso deficiencia en oligoelementos y alergia, debe contemplarse en el diagnóstico diferencial durante la fase crónica de la besnoitiosis bovina (Rostaher et al., 2010). Finalmente, cabe destacar que los quistes de *B. besnoiti* presentes en el vestíbulo vaginal deben diferenciarse de la vulvovaginitis pustular infecciosa que se produce durante la infección por el virus de la Rinotraqueitis Infecciosa Bovina.

3.7.4. Diagnóstico laboratorial

Actualmente, existen diferentes técnicas de diagnóstico para la detección de la infección por *B. besnoiti* en el ganado bovino. Existen tanto técnicas directas para la detección de parásito o de su ADN en tejidos (citología, histopatología, IHQ y PCR), como técnicas indirectas de diagnóstico serológico para la detección de anticuerpos específicos (revisado por Cortes et al., 2014).

3.7.4.1. Diagnóstico directo

En los animales crónicamente infectados, la existencia de quistes puede confirmarse mediante el examen de biopsias de piel por compresión en placas de triquineloscopía o incluso por histopatología, IHQ y PCR. En particular, la IHQ es un tipo de técnica histológica que se basa en la detección del parásito en cortes histológicos mediante anticuerpos específicos (Figura 9). Aunque su Esp es extremadamente elevada, es poco sensible para la detección de los animales con baja carga parasitaria ya que, generalmente, presentan un número bajo de quistes, al igual que sucede al emplear la histopatología y la compresión en placas de triquineloscopía. Por lo tanto, si éstos no se detectan en una biopsia, no puede descartarse la infección. Es por ello, que la toma de muestras juega un papel esencial en el diagnóstico directo de animales infectados. Estas siempre deben recogerse de las zonas de la piel con lesiones más evidentes.

El empleo de técnicas de PCR (tanto convencional como cuantitativa a tiempo real) puede resultar de utilidad, en ciertas ocasiones, para detectar y/o cuantificar el parásito no solo en tejidos sino también en sangre. La PCR convencional desarrollada hasta el momento, se basa en la amplificación de un fragmento de aproximadamente 231pb de la región ITS-1 del ADN y ha sido aplicada para la detección del ADN parasitario en biopsias de piel de animales infectados (Cortes et al., 2007b; Schares et al., 2011b). Además, esta técnica se ha empleado para comparar los aislados de *B. besnoiti* obtenidos en Alemania, España, Israel y Portugal, confirmándose una identidad genética del 100% (Schares et al., 2009). Por otra parte, Cortes et al. (2007b) y Schares et al. (2009)

desarrollaron una PCR cuantitativa a tiempo real que ha sido empleada, posteriormente, por Frey et al. (2013a, b) y Basso et al. (2013) para estudiar la distribución intraorgánica del parásito en animales con baja carga parasitaria, siendo las localizaciones más frecuentes el tracto respiratorio superior, el tracto reproductor distal de las hembras, la piel del cuello, los tendones y la fascia de las extremidades. Además, también ha sido empleada por Gutiérrez-Expósito et al. (2015) para detectar parasitemia en animales infectados. Por otra parte, se ha empleado también para estudiar el papel que juegan los vectores artrópodos en la transmisión y diseminación del parásito, si bien no ha permitido determinar si el parásito está vivo y tiene capacidad infectante. Así, se ha podido demostrar, bajo condiciones experimentales, la presencia de ADN de *B. besnoiti* en el aparato bucal de *S. calcitrans* tras la inoculación subcutánea de quistes o tras la ingestión de sangre de animales infectados (Liénard et al., 2013). Además, Gollnick et al. (2015) han demostrado, recientemente, la presencia de ADN de *B. besnoiti* en un ejemplar de *S. calcitrans* capturado directamente de un animal infectado durante la fase aguda de la enfermedad.

3.7.4.2. Diagnóstico indirecto

Las técnicas serológicas desarrolladas hasta el momento, se basan en la detección de anticuerpos específicos frente a *B. besnoiti,* y son las que se emplean en el diagnóstico rutinario de la besnoitiosis bovina. Entre las técnicas desarrolladas hoy en día se encuentran la IFI, la aglutinación, el Western blot y el ELISA, siendo la última la más empleada, tanto para diagnóstico como para realizar estudios epidemiológicos (Cortes et al., 2006a; Fernández-García et al., 2009a, 2010; Schares et al., 2010, 2013; Waap et al., 2011) (Tabla 2). Por otra parte, la IFI fue la primera técnica serológica desarrollada (Goldman y Pipano, 1983), y constituye en la actualidad una técnica de referencia para el desarrollo de otras técnicas (Shkap et al., 2002). Hasta la fecha, se han desarrollado diversas pruebas, basadas siempre en el empleo de taquizoítos que se usan de forma rutinaria en los diferentes laboratorios (Shkap et al., 2002; Cortes et al., 2006a; Fernández-García et al., 2009a; Schares et al., 2009; Lenfant et al., 2014). Sin embargo,

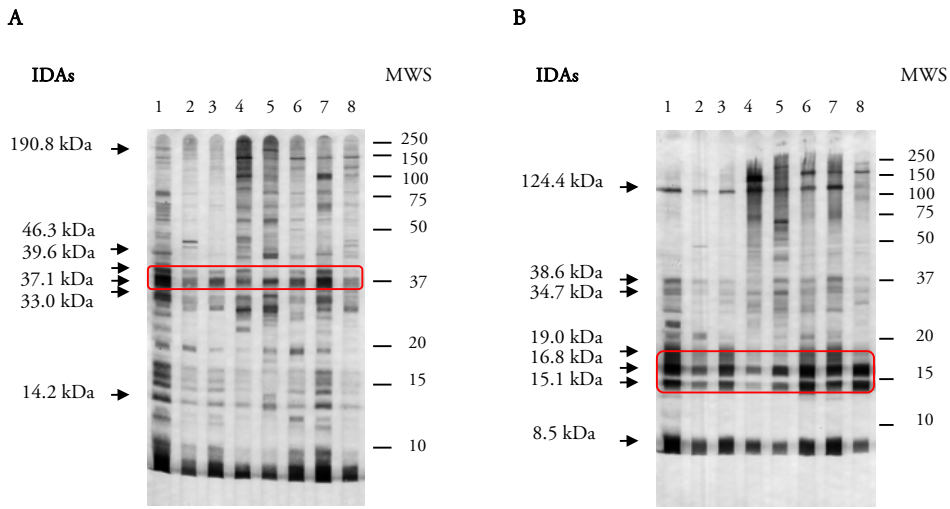
la Se y la Esp de cada una de ellas depende, principalmente, de la experiencia del observador a la hora de interpretar los resultados. En relación al Western blot, se han desarrollado pruebas basadas en extractos tanto de taquizoíto como de bradizoíto y se han empleado tanto en condiciones reductoras como no reductoras. En particular, las pruebas desarrolladas por Fernández-García et al. (2009a) y Schares et al. (2010), basadas en extracto del taquizoítos y bradizoítos en condiciones reductoras y no reductoras, respectivamente, han mostrado valores de Se y Esp cercanos al 100% (Figura 10) y, por tanto, han sido empleados en numerosos estudios como prueba de confirmación de los resultados obtenidos con otras técnicas serológicas (Schares et al., 2010, 2011a; Nasir et al., 2012; Gazzonis et al., 2014; Gutiérrez-Expósito et al., 2014). Cabe destacar que esta prueba no se utiliza de forma rutinaria para el diagnóstico de la infección debido su alto coste.

Tabla 2. Técnicas de diagnóstico serológico empleadas en la detección de anticuerpos específicos frente a *B. besnoiti*.

Técnica	Tipo de antígeno	Aislado	Condiciones	Se/Esp	Comercialización	Referencia
Aglutinación	Taquizoítos completos	Bb1Evora03	Fijados con formaldehído	97,2/99,3	-	Waap et al., 2011
IFI	Taquizoítos completos	-	En PBS	-	-	Goldmand y Pipano, 1983
		Bb-Israel	Fijados con formaldehído	-	-	Shkap et al., 2002
		Bb-Israel	Fijados con paraformaldehído	-	-	Cortes et al., 2006a
		BbSpain1	Fijados con formaldehído	-	-	Fernández-García et al., 2009a
		Bb1Evora03	En PBS	91,9/100	-	Schares et al., 2010
		-	Fijados con paraformaldehído	91,8/100		Lenfant et al., 2014
ELISA	Extracto soluble de taquizoítos	Bb1Evora03	Extracto obtenido por sonicación, eliminación de fracción insoluble	87/96.4	-	Cortes et al., 2006a
		BbSpain1		100/100	-	Fernández-García et al., 2010
	Extracto de taquizoíto enriquecido en proteínas de membrana	Bb1Evora03	Marcaje con biotina y purificación de extracto por cromatografía de inmunoafinidad	100/99,8	-	Schares et al., 2013
	Extracto total de taquizoíto	Bb-Israel	Extracto obtenido con Tritón X 100	-	-	Shkap et al., 1984
		-	-	91,8/96,8	PrioCHECK Besnoitia Ab (Termo Fisher Scientific)	Schares et al., 2011a
	-	-	-	-	INGEZIM BES 12.BES.K1 INGENASA	-
	-	-	-	-	ID SCREEN® BESNOITIA INDIRECT	-
Western blot	Extracto total de taquizoíto	Bb-Israel	Condiciones reductoras	-	-	Shkap et al., 2002
	Taquizoítos	BbSpain1	Condiciones reductoras	-	-	Fernández-García et al., 2009a
		Bb1Evora03	Condiciones no reductoras	91,3/96,4	-	Cortes et al., 2006a
				90/100	-	Schares et al., 2010
	Bradizoítos	-	Condiciones reductoras	-	-	Fernández-García et al., 2009a
		-	Condiciones no reductoras	90,3/99,5	-	Schares et al., 2010

Figura 10. Patrón de reconocimiento de antígenos de taquizoíto y bradizoíto de *B. besnoiti* (Fuente: Fernández-García et al., 2009a).

Patrón de reconocimiento de antígenos del extracto del taquizoíto (A) y del bradizoíto (B). Los antígenos inmunodominantes (IDAs) (bandas reconocidas por el 75% de los animales seropositivos) se muestran en la izquierda de cada imagen. El criterio de positividad se encuentra señalado en rojo: reconocimiento de antígeno de 37,1 kDa y de los antígenos de 15,1 y 16,8 kDa en los Western blot de taquizoíto y bradizoíto, respectivamente.



El diagnóstico serológico de los animales durante la fase crónica y que además presentan signos clínicos evidentes es sencillo, ya que la mayoría son seropositivos. Sin embargo, las pruebas serológicas desarrolladas hasta el momento presentan dos limitaciones.

En primer lugar, es difícil detectar animales infectados durante la fase aguda, ya que no ha pasado el tiempo suficiente para el desarrollo de anticuerpos específicos (revisado por Jacquiet et al., 2010). Además, existen animales con infección crónica (con y sin signos clínicos) con bajos niveles de anticuerpos que muchas veces no son detectables por las pruebas empleadas. En general, estos animales representan un porcentaje muy bajo de los animales infectados en brotes recientes (1-3%) (Fernández-García et al., 2009a;

Schares et al., 2009). Sin embargo, en áreas donde la enfermedad es endémica, pueden representar hasta un 17% de los casos, lo que constituye un riesgo muy importante para control de la enfermedad (Liénard et al., 2011; Gutiérrez-Expósito et al., 2015). Por otra parte, se han descrito problemas de Esp, que repercuten sobre los resultados de los estudios epidemiológicos y pueden tener consecuencias en los planes de control. De hecho, las tasas de seroprevalencia del 18,4% y del 18,8% descritas por Nasir et al. (2012) y Gazzonis et al. (2014) en Australia e Italia, usando las pruebas PrioCHECK Besnoitia Ab 2.0 y BbSALUVET ELISA 1.0 (Fernández-García et al., 2010), respectivamente, no pudieron ser confirmadas mediante Western blot. Esta limitación no es exclusiva del ELISA, ya que Uzêda et al. (2014) tampoco pudo confirmar por Western blot la seroprevalencia del 3,5% observada en ganado procedente de Brasil usando IFI. Estos hechos ponen en cuestión los resultados del ELISA descritos en los estudios serológicos llevados a cabo recientemente en Italia, Egipto, Grecia y Jordania, los cuales tampoco han sido confirmados por técnicas más específicas (Rinaldi et al., 2013; Ashmawy y Abu-Akkada, 2014; Papadopoulos et al., 2014; Talafha et al., 2015). Esta baja Esp puede deberse a la existencia de reacciones cruzadas con otros parásitos apicomplejos cercanos como *Sarcocystis* spp. y *N. caninum*. De hecho, se piensa que casi el 100% del ganado bovino está infectado con *S. cruzi* (Moré et al., 2011). Sin embargo, las reacciones cruzadas entre las especies de *Sarcocystis* que afectan a bovino (*S. cruzi, S. hominis, S. hirsuta* y *S. rommeli*) (Dubey et al., 2015) y *B. besnoiti* no se han estudiado. En relación a *N. caninum*, los estudios llevados a cabo por Shkap et al. (2002) pusieron de manifiesto que los anticuerpos frente a *N. caninum* reaccionaban con antígenos de *B. besnoiti* por IFI usando diluciones bajas, lo cual puede ser muy relevante ya que se han descrito prevalencias de *N. caninum* intra-rebaño e individuales del 80,6% y 23,2%, respectivamente, en España (Eiras et al., 2011). En este sentido, el nuevo ELISA desarrollado por Schares et al. (2013), basado en un extracto en el que los antígenos de taquizoítos de *B. besnoiti* que reaccionan de forma cruzada con anticuerpos específicos anti-*N. caninum* se eliminaron, muestra una Esp muy alta. Además, ha sido adaptado para diferenciar y confirmar los casos de besnoitiosis aguda y crónica. A pesar de sus

buenas características diagnósticas, la metodología de producción de antígeno conlleva muchos pasos, por lo que su uso a escala comercial es prácticamente inviable. Sin embargo, puede resultar de gran utilidad para realizar estudios de patogenia, así como de seguimiento serológico de infecciones experimentales.

Por otra parte, las técnicas serológicas desarrolladas hasta el momento no son capaces de diferenciar entre las diferentes especies de *Besnoitia* que afectan a ungulados. De hecho, se han descrito intensas reacciones cruzadas entre *B. besnoiti* y *B. tarandi* y entre *B. besnoiti* y *B. bennetti* (Gutiérrez-Expósito et al., 2012; Ness et al., 2012). Sin embargo, esta limitación ha permitido aplicar las técnicas serológicas para estudiar el papel de los rumiantes silvestres en la epidemiología de la besnoitiosis bovina. Un ejemplo son los estudios llevados a cabo por Gutiérrez-Expósito et al. (2012, 2013) que han sugerido que los ciervos y corzos son susceptibles a la infección por *Besnoitia* spp., si bien no han permitido identificar la especie de *Besnoitia* presente en estos animales.

Otra cuestión de suma importancia es la comparación de resultados diagnósticos entre diferentes laboratorios, la cual se debe realizar con precaución, puesto que las técnicas para el diagnóstico de la besnoitiosis bovina no están estandarizadas entre los diferentes centros, siendo los resultados poco comparables. Por lo tanto es importante que se realicen estudios inter-centro de comparación de pruebas, por lo menos en el caso de las técnicas de elección rutinaria para el diagnóstico de la infección en ganado bovino, con el fin de homogeneizar las técnicas, hacer comparables los diferentes estudios y establecer protocolos de diagnóstico consensuados. En este sentido, la estandarización de las técnicas serológicas que se emplean de forma rutinaria en el diagnóstico de la neosporosis bovina en EE.UU. y Europa ya se ha llevado a cabo y las características diagnósticas de las pruebas evaluadas, han sido recientemente actualizadas (Von Blumröder et al., 2004; Wapenaar et al., 2007; Álvarez-García et al., 2014b). Un abordaje similar se ha llevado a cabo con la técnica de ELISA comercial PrioCHECK Besnoitia, la cual se ha validado y estandarizado tras varios estudios serológicos (Schares et al., 2010; Nasir et al., 2012).

3.8. Control

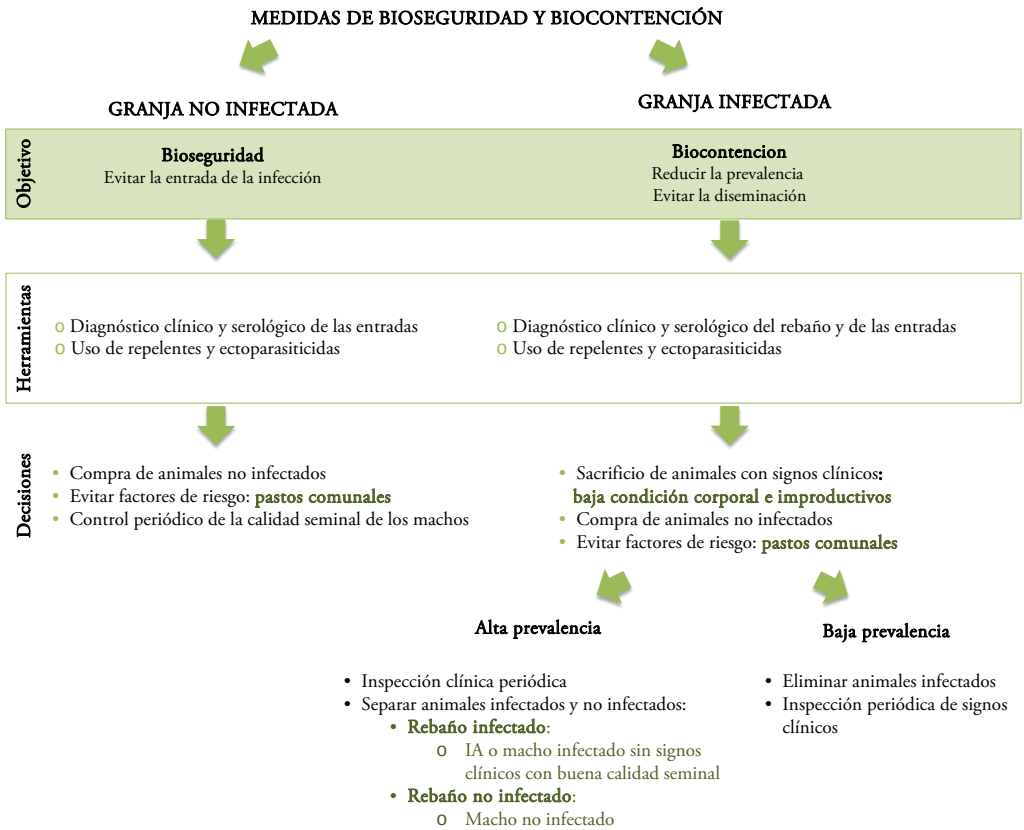
El control de la besnoitiosis bovina no es fácil ya que no existen fármacos ni vacunas eficaces disponibles, y la diseminación de la enfermedad está asociada a prácticas de manejo de riesgo que favorecen la misma. Por lo tanto, las medidas de bioseguridad junto con un diagnóstico precoz juegan un papel crucial en el control de la enfermedad. Éste debería tener dos objetivos principales: por un lado, evitar la entrada de la infección al rebaño (bioseguridad) y, por otro, evitar su diseminación dentro del mismo (biocontención) (Figura 11).

3.8.1. Bioseguridad y biocontención

Antes de implementar un programa de control es necesario conocer el estatus sanitario de la explotación. En las zonas libres de la enfermedad es primordial evitar la entrada de la infección (bioseguridad), ya que los nuevos brotes ocurren tras la introducción de animales portadores (Basso et al., 2013). Por otra parte, en zonas donde la enfermedad es endémica deberían evitarse en lo posible las medidas de manejo que puedan favorecer la transmisión y diseminación (biocontención), como es el uso de pastos comunales. Si esto no es posible, todos los animales deberían ser examinados al final de la temporada de pastoreo, hecho que coincide habitualmente con el periodo de máxima actividad de los insectos potenciales transmisores. Una vez que la infección entra en un rebaño, los animales infectados deberían estar separados de los sanos o alternativamente, ser eliminados tomando en consideración la presencia de signos clínicos y parámetros reproductivos, ya que, salvo en granjas con baja prevalencia, la eliminación de todos los animales infectados de un rebaño, no es posible (revisado por Álvarez-García et al., 2013). Una estrategia conservadora, y que a largo plazo parece ser la mejor opción, consiste en mantener un equilibrio entre el sacrificio selectivo y los parámetros productivos, ya que la seroprevalencia puede ser alta en un rebaño infectado (Bigalke, 1968; Kumi-Diaka et al., 1981). Por ello, sólo los animales afectados más gravemente e improductivos (machos estériles y hembras con baja condición corporal) han de ser reemplazados por animales sanos y seronegativos, que deberán ser mantenidos separados de los animales infectados.

En el caso de los sementales, es esencial realizar un control periódico de la calidad seminal ya que es posible el desarrollo de infertilidad. Así, los sementales seropositivos y fértiles deben mantenerse con el rebaño infectado, en el que puede ser también útil la implantación de un protocolo de inseminación artificial. Por el contrario, los sementales seronegativos deberán estar en contacto tan sólo con los animales sanos.

Figura 11. Control de la besnoitiosis bovina.



Las condiciones de manejo intensivo (o semi-extensivo), donde se lleva a cabo tanto la inseminación artificial como una monitorización de los parámetros productivos y no se realiza pastoreo, favorecen la posibilidad de separar animales sanos de aquellos que deberán ser sustituidos. Por tanto, la posibilidad de eliminar la enfermedad es mayor. Bajo condiciones de manejo extensivo, sin la posibilidad de analizar repetidamente a los individuos, separar en grupos y evitar la transmisión por insectos picadores, se puede conseguir una disminución en la prevalencia de la enfermedad, pero la erradicación es un objetivo prácticamente imposible y debemos contar con la aparición de signos clínicos periódicamente. Sin embargo, la disminución de la gravedad de los signos clínicos en el rebaño puede ser un objetivo razonable, que se puede lograr con la inspección visual periódica de los animales para identificar nuevos casos y eliminar los animales infectados.

3.8.2. Quimioterapia e inmunoprofilaxis

El desarrollo de vacunas y/o tratamientos eficaces parecen ser las opciones idóneas para el control de la enfermedad así como para evitar su entrada y diseminación. Respecto a la vacunación, actualmente no existen vacunas autorizadas en Europa. Por el contrario en Sudáfrica, durante los años 70 se desarrolló una vacuna viva basada en un aislado atenuado de ñu, capaz de evitar el desarrollo de signos clínicos. Sin embargo, la aplicación de esta vacuna no prevenía la aparición de portadores subclínicos (Bigalke et al., 1974). En Israel, en la actualidad se vacuna de forma rutinaria todos los sementales con un aislado atenuado (procedente de un toro infectado naturalmente) para proteger a los animales frente al desarrollo de signos clínicos. Sin embargo, no se ha realizado aún ningún estudio de eficacia de esta formulación vacunal y se desconoce su seguridad (Pipano, 1997; revisado por Cortes et al., 2014).

En relación al tratamiento, desafortunadamente no se ha desarrollado ningún fármaco eficaz y específico frente a la besnoitiosis bovina. La utilidad residirá en evitar la proliferación del taquizoíto, responsable de la fase aguda de la enfermedad, para, de esta forma, evitar la diseminación intraorgánica y el acantonamiento del parásito y, por tanto, la formación de los quistes tisulares (revisado por Montoya y Liesenfeld, 2004). Por el

contrario, el tratamiento de animales en fase crónica no tiene ningún efecto beneficioso, posiblemente debido a la limitada accesibilidad del fármaco al interior de los quistes tisulares (Pols, 1960; Rostaher et al., 2010). Los escasos estudios que se han realizado, tanto en modelos *in vitro* como en infecciones experimentales en jerbos, no han arrojado resultados prometedores (Shkap et al., 1985, 1987) con la excepción de varios fármacos probados en ensayos *in vitro* como son la arilimidamida y derivados de la nitozoxanida, valorados en estudios recientes (Cortes et al., 2007a, 2011).

Capítulo III

Justification and objectives

Bovine besnoitiosis is an emerging disease of cattle in Europe, caused by the cyst forming apicomplexan parasite *B. besnoiti.* Although low mortality rates are reported (up to 10%), it is a chronic and a debilitating disease responsible for severe economic losses mainly due to poor body condition and sterility in bulls (reviewed by Álvarez-García et al., 2014c). Since there are no efficient treatments or vaccines available, the control of bovine besnoitiosis relies solely on accurate diagnosis (clinical inspection and serological diagnosis) coupled with herd management in order to detect both, acute and chronically infected animals with and without pathognomonic visible tissue cysts. In Spain, it has been demonstrated high seroprevalence rates in areas where the disease is endemic (Álvarez-García et al., 2014a; Gutiérrez-Expósito et al., 2014). Moreover, new outbreaks of the disease have been recently reported and nowadays its spread goes on.

A wide number of serological methods including IFAT, ELISA, Western blot and direct agglutination tests have been developed to date (Cortes et al., 2006a; Fernández-García et al., 2009a, 2010; Schares et al., 2010, 2013; Waap et al., 2011). However, a gold standard technique has not yet been established, and comparable tests are urgently needed in order to accurately diagnose *B. besnoiti* infected cattle in disease outbreaks and for animal trade purposes. Moreover, they are necessary to carry out prevalence and incidence studies to determine the impact of the disease and, thus, implement common control strategies in affected countries as well as in those at risk of acquiring the disease.

However, two main limitations of serological diagnosis have been evidenced throughout several studies. First, *B. besnoiti* infected cattle may go unnoticed by serological tests, as antibody levels may decrease below the detection limits of the assays in chronically infected animals as it occurs in other Sarcocystidae infections (e.g. *N. caninum* infections; Dubey and Schares, 2006) (Fernández-García et al., 2010; Schares et al., 2010; Gutiérrez-Expósito et al., 2015). This issue represents an important risk for disease spread, since new outbreaks are associated to animal trade from highly prevalent areas into regions free of the infection (reviewed by Álvarez-García et al., 2013). Furthermore, recent studies have evidenced that Sp is also compromised using ELISAs

and IFAT tests, which may cause a high proportion of false-positive results (Schares et al., 2011a; Nasir et al., 2012; Gazzonis et al., 2014; Uzêda et al., 2014). Moreover, *B. besnoiti* and another *Besnoitia* species affecting wild ungulates (*B. tarandi)* cannot be differentiated by using these assays (Gutiérrez-Expósito et al., 2012). In this sense, a previous study has reported the presence of anti-*Besnoitia* spp. specific antibodies in serum samples from roe deers and red deers from the Spanish Pyrenees (an area where the disease is endemic) (Gutiérrez-Expósito et al., 2013). Based on these issues, the use of a confirmatory Western blot is esential for an accurate diagnosis (Schares et al., 2009). Unfortunately, it is an expensive and time-consuming tool and it is only available in some specialized laboratories. In this sense, there is an urgent need of developing more sensitive and specific ELISA assays for their use in both, diagnosis and epidemiological studies. However, first, the origin of the false-positive reactors previously reported needs to be addressed. Related to this issue, the existence of cross-reactions with related parasites has been suggested, since cross-reactivity at low IFAT dilutions was found between two sera showing high *N. caninum* antibody levels and *B. besnoiti* antigens (Shkap et al., 2002). Nevertheless, the existence of cross-reactive antigens between *B. besnoiti* and other apicomplexan parasites, such as *Sarcocystis* spp. remains to be studied.

In this scenario, the aim of the present Doctoral Thesis was to investigate new strategies for the improvement of bovine besnoitiosis serological diagnosis. To this end, first, a comparative study of the serological tests available in Europe was carried out in order to obtain comparable results among all the laboratories and to establish a common diagnostic procedure. Moreover, the origin of the false-positive results in *B. besnoiti* serological assays was investigated in order to establish a more appropriate panel sera to be employed in future validations assays to improve the Sp of the tests. Then, new diagnostic targets for a specific and sensitive diagnosis of bovine besnoitiosis were identified and their diagnostic value was evaluated by using two different approaches: *i)* proteomic studies were carried out to identify *B. besnoiti* specific and immunogenic proteins, and MABs directed against *B. besnoiti* antigens were also developed and characterized. Moreover *ii)* a new *B. besnoiti* extract based on *B. besnoiti* lyophilized whole

tachyzoites was obtained and a new ELISA test was developed to diagnose *Besnoitia* spp. infection in bovids and wild ruminants.

- **Objective 1**

Comparison of the serological tools employed in the diagnosis of *B. besnoiti* infection in bovines.

The aim of this study was to compare the performance of the serological tests available in Europe in a multi-centred study in order to establish a gold standard technique and to adopt common diagnostic procedures for use in disease control. For this purpose, a coded panel of 241 well-characterized sera from infected and non-infected bovines was provided by all participants working on bovine besnoitiosis (SALUVET-Madrid, Friedrich Loeffler Institut (FLI)-Wusterhausen, Ecole Nationale Vétérinaire (ENV)-Toulouse and Institute of Parasitology of Berne (IPB)-Berne). Two different definitions of a gold standard were used: *i)* the result of the majority of tests ('Majority of tests') and *ii)* the majority of test results plus pre-test information based on clinical signs ('Majority of tests plus pre-test info'), and the diagnostic performance of all tests was investigated.

- **Objective 2**

Investigation of the origin of *B. besnoiti* false-positive ELISA results.

Since recent studies have reported that routinely used ELISAs may raise a high number of false-positive results, ELISA cross-reactions between *B. besnoiti* and anti-*N. caninum* and/or *Sarcocystis* spp. specific antibodies were investigated, since both Sarcocystidae infections are highly prevalent in cattle worldwide. For this purpose, a panel sera composed of *B. besnoiti* non-infected cattle with either an ELISA negative or positive result and seropositive infected cattle was analyzed. Additional reference sera from *S. cruzi* and *N. caninum* infected cattle were also tested by *Sarcocystis* spp. cystozoite based IFAT and Western blot and *N. caninum* tachyzoite-based ELISA and Western blot. Then, the association between *B. besnoiti* false-positive ELISA results and the presence and level of anti-*Sarcocystis* spp. and/or -*N. caninum* specific antibodies was studied.

- **Objective 3**

Identification of new *B. besnoiti* diagnostic targets and development of a new serological assay.

The aim of this objective was to identify specific *B. besnoiti* diagnostic targets in order to avoid serological cross-reactions with related apicomplexan parasites. For this issue, first, proteomics studies were carried out in order to identify valuable diagnostic targets. MABs were also developed against *B. besnoiti* tachyzoite stage and were further characterized. Finally, a *B. besnoiti* lyophilized whole tachyzoite antigen was obtained and a new ELISA test was developed to diagnose besnoitiosis in bovids and wild ruminants.

- Sub-objective 3.1

First 2-DE approach towards characterising the proteome and immunome of *B. besnoiti* in the tachyzoite stage.

The aim of this study was to conduct a proteomics study to describe for the first time the proteome and immunome of the tachyzoite stage of *B. besnoiti* using two-dimensional gel electrophoresis (2-DE SDS-PAGE) and 2-DE immunoblot. For this purpose, pooled sera from *B. besnoiti* naturally infected cattle were employed in the immunoblot. Additionally, abundant spots were selected for protein identification by MALDI-TOF/MS and bioinformatics. Finally, cross-reactive antigens were also studied with pooled sera from *N. caninum* infected cattle.

- Sub-objective 3.2

Proteomics reveals differences in protein abundance and highly similar antigenic profiles between *B. besnoiti* and *B. tarandi*.

A DIGE approach and MALDI-TOF/MS were used to compare the proteomes of *B. besnoiti* and *B. tarandi,* and explore differences in protein abundance between tachyzoite extracts from both species. Immunoproteomes were also compared using 2-DE immunoblotting with polyclonal sera from experimentally infected rabbits. Finally, spots that showed differences in abundance were further analyzed by MALDI-TOF/MS.

- Sub-objective 3.3

Development and characterization of MABs against *B. besnoiti* tachyzoites.

Herein eight MABs were developed against a whole- and a membrane enriched- *B. besnoiti* tachyzoite extracts and were further characterized. To this end, co-localization and TEM studies were carried out, isotyping was performed and genus-, species- and stage- specificity was checked in the tachyzoites of the closely related protozoan *N. caninum*, *T. gondii* and *B. tarandi,* in *Sarcocystis* spp. cystozoites and in *B. besnoiti* bradyzoites by Western blot.

- Sub-objective 3.4

A new ELISA test to diagnose *Besnoitia* spp. infection in bovines and wild ruminants renders unnecessary the use of a confirmatory test.

In the present study, a *B. besnoiti* lyophilized whole tachyzoite antigen was obtained and was employed for the development of a new ELISA test (BbSALUVET ELISA 2.0) that was validated with cattle sera under worst-case scenario for an accurate diagnosis of bovine besnoitiosis. For this purpose, false-positive and false-negative soluble tachyzoite extract based BbSALUVET ELISA 1.0 reactors were overrepresented and Western blot was considered to be the reference test. One commercial test (PrioCHECK Besnoitia Ab 2.0 that employs whole tachyzoite extract) and a recently developed membrane enriched ELISA (APure-BbELISA) were also tested in order to update their diagnostic characteristics. In addition, BbSALUVET ELISA 2.0 was also adapted to wild ruminant sera and the diagnostic characteristics were estimated.

Capítulo IV

Objetivo 1. Comparación de las técnicas serológicas empleadas en el diagnóstico de la besnoitiosis bovina.

La besnoitiosis bovina es una enfermedad crónica y debilitante que ocasiona importantes pérdidas económicas a nivel mundial. En la actualidad no existen fármacos ni vacunas eficaces frente a la enfermedad y, por tanto, las medidas de control se deben basar en la detección de animales infectados para reducir la prevalencia en zonas endémicas y evitar la entrada de la enfermedad en zonas libres a través de la compra de animales infectados. En relación a las actuales técnicas de diagnóstico serológico de la besnoitiosis bovina que se están empleando en diferentes laboratorios, éstas no han sido estandarizadas con una población de referencia común y de momento no se ha determinado la prueba de referencia. Por ello, el objetivo de este trabajo ha sido realizar un estudio comparativo en el que se han incluido las diferentes técnicas de diagnóstico serológico empleadas por cuatro laboratorios europeos [SALUVET, Madrid (España), FLI, Wusterhausen (Alemania), ENV, Toulouse (Francia) e IPB, Berna (Suiza)] con el fin de *i)* establecer las mejores condiciones de la técnica de Western blot; *ii)* determinar la Se y Esp de cada una de las pruebas diagnósticas evaluadas; *iii)* validar las tres únicas pruebas ELISA comerciales existentes en el mercado y *iiii)* determinar la prueba de referencia. Para la realización de este trabajo, los laboratorios participantes han analizado un total de 241 sueros bovinos procedentes de animales con o sin signos clínicos asociados a la infección por *B. besnoiti* mediante las siguientes técnicas serológicas: dos pruebas IFI, una prueba ELISA *in house* desarrollada por el grupo de investigación SALUVET, tres pruebas ELISA comerciales (INGEZIM BES 12.BES.K1 INGENASA, PrioCHECK Besnoitia Ab V2.0, ID Screen Besnoitia indirect IDVET) y siete tipos diferentes de Western blot (evaluando diferentes condiciones -reductoras y no reductoras- y diferentes preparaciones antigénicas -taquizoíto y bradizoíto-). De acuerdo con los resultados obtenidos cualquiera de las pruebas ELISA evaluadas podría emplearse tanto en estudios de prevalencia como para determinar el estatus sanitario de una granja en relación a esta infección, gracias a la elevada Se y Esp de todas ellas. Sin embargo, se ha recomendado el

empleo *a posteriori* de cualquier prueba Western blot con valores de Se y Esp cercanos al 100% en los siguientes casos: *i)* para confirmar el diagnóstico en el caso de animales con resultados dudosos en la prueba ELISA; *ii)* en el diagnóstico de la infección en las nuevas incorporaciones en rebaños libres de la infección y *iii)* en el diagnóstico de la infección en animales valiosos antes de su sacrificio selectivo. Además, en relación a esta técnica, se ha podido observar que tanto el Western blot basado en extracto del taquizoíto, como el basado en bradizoíto, no presentan diferencias significativas en términos de Se y Esp.

An inter-laboratory comparative study of serological tools employed in the diagnosis of *Besnoitia besnoiti* infection in bovines

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Abstract

Bovine besnoitiosis is considered an emerging chronic and debilitating disease in Europe. Many infections remain subclinical, and the only sign of disease is the presence of parasitic cysts in the sclera and conjunctiva. Serological tests are useful for detecting asymptomatic cattle/sub-clinical infections for control purposes, as there are no effective drugs or vaccines. For this purpose, diagnostic tools need to be further standardized. Thus, the aim of this study was to compare the serological tests available in Europe in a multi-centred study. A coded panel of 241 well-characterized sera from infected and non-infected bovines was provided by all participants (SALUVET-Madrid, FLI-Wusterhausen, ENV-Toulouse, IPB-Berne). The tests evaluated were as follows: an in house ELISA, three commercial ELISAs (INGEZIM BES 12.BES.K1 INGENASA, PrioCHECK Besnoitia Ab V2.0, ID Screen Besnoitia indirect IDVET), two IFATs and seven Western blot tests (tachyzoite and bradyzoite extracts under reducing and non-reducing conditions). Two different definitions of a gold standard were used: (i) the result of the majority of tests ('Majority of tests') and (ii) the majority of test results plus pre-test information based on clinical signs ('Majority of tests plus pre-test info'). Relative to the gold standard 'Majority of tests', almost 100% sensitivity (Se) and specificity (Sp) were obtained with SALUVET-Madrid and FLI-Wusterhausen tachyzoite- and bradyzoite-based Western blot tests under non-reducing conditions. On the ELISAs, PrioCHECK Besnoitia Ab V2.0 showed 100% Se and 98.8% Sp, whereas ID Screen Besnoitia indirect IDVET showed 97.2% Se and 100% Sp. The in house ELISA and INGEZIM BES 12.BES.K1 INGENASA showed 97.3% and 97.2% Se; and 94.6% and 93.0% Sp, respectively. IFAT FLI-Wusterhausen performed better than IFAT SALUVET-Madrid, with 100% Se and 95.4% Sp. Relative to the gold standard 'Majority of test plus pre-test info', Sp significantly decreased; this result was expected because of the existence of seronegative animals with clinical signs. All ELISAs performed very well and could be used in epidemiological studies; however, Western blot tests performed better and could be employed as *a posteriori* tests for control purposes in the case of uncertain results from valuable samples.

Keywords: *Besnoitia besnoiti;* Diagnosis; Enzyme-linked immunosorbent assay; Indirect fluorescent antibody test; Western blot; Comparative study.

1. Introduction

Bovine besnoitiosis is a cattle disease caused by the cyst-forming apicomplexan parasite *Besnoitia besnoiti* (Marotel, 1912). This severe but usually non-fatal disease has been previously described in Sub-Saharan Africa, Asia and western and central Europe (EFSA, 2010; reviewed by Jacquiet et al., 2010).

Two asexual and infective stages of this parasite develop in cattle, which act as the intermediate host: the fast-replicating tachyzoites, which multiply by endodyogeny in endothelial cells, fibroblasts and macrophages of blood vessels, and the slow-dividing bradyzoites, which gather into macroscopic cysts inside cells of the subcutaneous connective tissue, upper respiratory tract, testes, scleral conjunctiva and mucous membranes of the *vestibulum vaginae* and vagina (Basson et al., 1970; Nobel et al.,1977; Rostaher et al., 2010). In the acute stage of the disease (anasarca stage), bovine besnoitiosis is characterized by hyperthermia, photophobia, ocular and nasal discharge, lymphadenitis, subcutaneous oedema (anasarca), lameness and orchitis. During the chronic stage, a progressive thickening, hardening and folding of the skin, hyperkeratosis and alopecia are noticed in heavily infected animals (Bigalke and Prozesky, 1994).

Recent epidemiological data confirm an increasing number of cases and a geographic expansion of the disease in cattle herds in Europe. At present, bovine besnoitiosis is considered an emerging disease (EFSA, 2010). Unfortunately, many aspects of the epidemiology and transmission of the infection are still unknown; this difficulty hinders the implementation of appropriate control measures, as there are no effective drugs or vaccines available at present. It is suspected that *B. besnoiti*, like other *Besnoitia* spp., has a heteroxenous life cycle, with domestic and wild cats acting as the definitive host (Wallace and Frenkel, 1975; Dubey, 1977; Rommel, 1978). However, in later studies, the persistence of the parasite and the shedding of oocysts could not be demonstrated in experimentally infected cats with tissue cysts containing bradyzoites and obtained from naturally infected cattle. (Diesing et al., 1988; Basso et al., 2011).

Nevertheless, the most likely path of transmission would be direct contact from infected to healthy animals through lacerations and mechanical transmission by blood-sucking arthropods (reviewed by Jacquiet et al., 2010).

Current control measures should focus on the detection of infected animals to reduce the prevalence in endemic areas and to avoid the entrance of the disease into non-endemic areas through the purchase of infected animals. The diagnosis protocol should include a combination of clinical inspection and serology to detect both chronically infected animals with visible tissue cysts and sub-clinically infected cattle, which represent the majority of affected herds (Fernández-García et al., 2010). In the past, IFAT and ELISA tests were developed and used in the first epidemiological studies on the subject (Goldman and Pipano, 1983; Janitschke et al., 1984; Shkap et al., 1984). Other sensitive and specific serological tests such as IFATs (Cortes et al., 2006a; Fernández-García et al., 2009a; Schares et al., 2010), commercial and in house ELISAs (Cortes et al., 2006a; Fernández-García et al., 2010; Schares et al., 2011a) and Western blots (Cortes et al., 2006a; Fernández-

García et al., 2009a; Schares et al., 2010) have been developed recently; however, a gold standard technique has not yet been established, and comparable tests are needed to carry out prevalence studies in different countries to determine the impact of the disease and thus implement effective control programmes.

Therefore, the aim of this study was to compare all of the serological tests available in Europe to detect anti-*B. besnoiti*-specific antibodies [two IFAT tests, one in house ELISA, three commercial ELISAs (INGEZIM BES 12.BES.K1 INGENASA, PrioCHECK Besnoitia Ab V2.0, ID Screen Besnoitia indirect IDVET) and seven Western blot tests] in a multi-centred study to establish a gold standard technique and adopt common diagnostic procedures for use in disease control.

2. Material and Methods

2.1. Sera and experimental design

A coded panel of 241 bovine sera (n = 181 cows, n = 13 bulls, n = 3 calf and n = 44 age undetermined) from well-documented naturally infected cattle and from non-infected cattle was tested in a ring trial by four laboratories in Europe (SALUVET group-UCM, Madrid, Spain; Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Institute of Epidemiology, Wusterhausen, Germany; Laboratoire de Parasitologie, Ecole Nationale Vétérinaire (ENV), Toulouse, France and Institute of Parasitology, University of Berne (IPB), Berne, Switzerland). Sera were provided by all participants in the study and sent to the SALUVET group in Madrid.

The sera were coded, and aliquots were sent out to the partnered laboratories for a blinded analysis.

The serum panel consisted of:

1. sera from animals naturally infected with *B. besnoiti* collected from herds with a previous history of besnoitiosis
   a. with clinical signs
      i. corresponding to the acute febrile stage (n = 13)
      ii. corresponding to the acute anasarca stage (n = 1)
      iii. corresponding to the chronic stage:
      • visible tissue cysts in the scleral conjunctiva and/or on the mucous membranes of the *vestibulum vaginae*: 31 samples
      • skin disorders (lichenification, hyperkeratosis, scars in teats and alopecia): 22 samples
      • skin disorders and visible tissue cysts: 15 samples
   b. without clinical signs and seropositive: 42 samples
2. 80 sera from seronegative cows that came from an area where besnoitiosis has not been described before.
3. 30 and three sera samples from animals naturally infected with *Neospora caninum* and *Toxoplasma gondii*, respectively, and four samples from cows from an area not endemic for bovine besnoitiosis and seropositive for both of these other infections by ELISA were included in the experiment to study cross-reactions with other apicomplexan parasites.

The tests evaluated were as follows: an in house ELISA developed by the SALUVET group (Fernández-García et al., 2010), three commercial ELISAs (PrioCHECK Besnoitia Ab V2.0, INGEZIM BES 12.BES.K1 INGENASA, ID Screen Besnoitia indirect IDVET), two

IFATs (developed by Fernández-García et al., 2009a and Schares et al., 2010) and seven Western blot tests (tachyzoite or bradyzoite extracts under reducing or non-reducing conditions) (Fernández-García et al., 2009a; Schares et al., 2010; Liénard et al., 2011). Moreover, PrioCHECK Besnoitia Ab V2.0 ELISA was carried out by every partner to evaluate the reproducibility of the test (Table 1).

2.2. Cell culture and antigen purification

Besnoitia besnoiti tachyzoites from BbSp-1 isolate (Fernández-García et al., 2009b) were grown in a Marc-145 cell monolayer with DMEM supplemented with 5% foetal calf serum and were purified following a previously described procedure (Fernández-García et al., 2009b). Bradyzoites were released by trypsinization of a skin biopsy from the skin of the neck and the vulva that contained numerous macroscopic tissue cysts from a chronically infected cow, following a previously described method (Fernández-García et al., 2009b). These zoites were employed as antigens in all serological tests developed by SALUVET-Madrid.

The FLI-Wusterhausen laboratory employed the Bb1Evora03 strain of *B. besnoiti* (Cortes et al., 2006c), which was maintained in Vero cell cultures and purified as previously described (Schares et al., 2010). Purified tachyzoites were used to prepare IFAT slides or pelleted by centrifugation at 1,300 g for 10 min and frozen at -80 ºC until they were used for immunoblots. Bradyzoites were isolated and purified following the method described by Schares et al. (2010). Frozen purified tachyzoites and bradyzoites were employed as antigens in Western blots under non-reducing conditions.

In ENV-Toulouse, tachyzoites from a *B. besnoiti* strain isolated in an area of emergence in southern France were maintained on Vero cells as described previously (Cortes et al., 2006c). Tachyzoites were purified as previously described (Liénard et al., 2011) to carry out a tachyzoite- based Western blot under non-reducing conditions.

Table 1. Tests performed by each laboratory.

Tests	WB under non-reducing conditions		WB under reducing conditions		IFAT	ELISA			
Partners	Tachyzoite	Bradyzoite	Tachyzoite	Bradyzoite		In house	INGENASA	IDVET	PRIONICS
SALUVET-Madrid	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
FLI-Wusterhausen	Yes	Yes			Yes				Yes
IPB-Bern									Yes
ENV-Toulouse	Yes								Yes

2.3. IFAT

SALUVET-Madrid

Sera were analysed by IFAT in double serial dilutions starting at 1:50 by a previously described method (Fernández-García et al., 2009a). Sera with unbroken tachyzoite membrane fluorescence at titres ≥ 1:100 were considered seropositive. Sera with membrane fluorescence titres ≤ 1:50 were considered seronegative.

FLI-Wusterhausen

At FLI-Wusterhausen, sera were analysed by IFAT as previously described (Schares et al., 2010). IFAT titres ≥ 1:200 were considered positive.

2.4. ELISA

In house ELISA

Coating of the plates and the test were performed essentially as described by Fernández-García et al. (2010). Serum samples were analysed in duplicate, and the mean value of the optical density (OD) was converted into a relative index per cent (RIPC) by the following formula: RIPC = (OD405 sample) OD405-negative control)/(OD405-positive control) OD405-negative control) × 100. Samples with a RIPC ≥ 9 were considered positive. For the test, suitable positive- and negative-control sera were used (Fernández-García et al., 2010).

Commercial ELISAs

For testing and interpretation of results, the manufacturer's instructions were followed. Test results were expressed as sample/positive control (S/P) ratios (ID Screen Besnoitia indirect IDVET test: < 60% negative; 60-70: doubtful; > 70% positive) or RIPC (INGEZIM BES 12.BES.K1 INGENASA test: < 6: negative; 6-9: doubtful; > 9: positive) based on positive- and negative-control sera. OD values were described as a percentage of the OD readings of a high positive control [called per cent positivity (PP); PrioCHECK Besnoitia Ab 2.0: < 15: negative; > 15: positive].

2.5. Western blot

SALUVET-Madrid

Coated membranes and the immunoblot were performed following the previously described method (Fernández-García et al., 2009a) in 12.5% polyacrylamide gel. Dithiothreitol (DTT) was added before sonication in an ultrasonic bath under reducing conditions only. For the tachyzoite Western blot performed under reducing conditions, sera recognizing the 37.1 kDa antigen were considered positive. In the bradyzoite-based Western blot, sera recognizing 16.1, 15.8 and 8.5 kDa antigens were considered positive. For the tachyzoite-based Western blot performed under non-reducing conditions, three main antigenic reactivity areas were described: area I (72.5, 58.9 and 51.4 kDa), area II (38.7, 31.8 and 28.5 kDa) and area III (23.6, 19.1, 17.4 and 14.5 kDa). Sera with at least three bands in at least two of the three areas described were considered positive. For the bradyzoite-based Western blot performed under non-reducing conditions, the three main antigenic reactivity areas considered were as follows: area I (153.6 kDa), area II (34.2, 31.8, 27.7 and 24.8 kDa) and area III (18.5, 17.0, 16.1 and 14.4 kDa). The presence of at least three

bands in at least two of the three areas described was recorded as a positive result.

FLI-Wusterhausen

The coated membranes and the immunoblots were performed as previously described (Schares et al., 2010). Ten immunogenic antigens in the tachyzoite-based Western blot (45, 40, 37, 34, 30, 27, 22, 17, 16 and 15 kDa) and in the bradyzoite-based Western blot (41, 36, 33, 28, 26, 24, 23, 22, 20 and 18 kDa) were analysed. Sera reactive to at least four antigens in each case were regarded as positive.

ENV-Toulouse

The coated membranes and the immunoblots were performed as previously described (Liénard et al., 2011). Three main antigenic reactivity areas are described (Cortes et al., 2006a): area I, 12-20 kDa; area II, 23-38 kDa and area III, 60-90 kDa. The minimal criterion for seropositivity was the recognition of four bands in area I, four bands in area II and another four bands in area III.

2.6. Analysis of data

Test agreement (expressed as Kappa-values) and sensitivity and specificity, including 95% confidence intervals (95% CI), were calculated using WinEpiscope 2.0 (Thrusfield et al., 2001).

Two different definitions of a gold standard were used to calculate the diagnostic characteristics of the tests. The first gold standard was defined by the decision of the majority of the tests ('Majority of tests'). If equal numbers of tests returned positive and negative results, the sample was regarded as doubtful and discarded (positive n = 110; negative n = 130; doubtful n = 1). Although the PrioCHECK Besnoitia Ab 2.0 ELISA was carried out by every partner, when the results were included in the gold standard, only the results obtained by the majority of the laboratories were taken into account.

The second gold standard was defined according to both the decision of the majority of the tests and the pre- test information ('Majority of test plus pre-test info'). A sample was considered positive when the animals had shown clinical signs compatible with the acute or chronic stage of the disease and/or was seropositive according to the majority of the tests. Consequently, seronegative animals with clinical signs and subclinical carriers were regarded as positive. Only sera negative by the majority of the tests from cattle without any sign compatible with bovine besnoitiosis were included as negative (positive n = 123; negative n = 117; doubtful n = 1).

TG-ROC analysis was carried out with respect to the gold standard 'Majority of tests' (Greiner et al., 1995). The reproducibility of the PrioCHECK ELISA 2.0 was calculated as the coefficient of variation (CV) (Jacobson, 1998).

3. Results

3.1. Sensitivity (Se) and specificity (Sp) of tests according to 'Majority of tests' and 'Majority of tests plus pre-test info' gold standard

Se and Sp values were calculated for each ELISA, IFAT test and Western blot on the basis of the cut-offs recommended by each laboratory. Relative to the gold standard 'Majority of tests', almost 100% Se and Sp were obtained with FLI-

Wusterhausen and SALUVET-Madrid tachyzoite- and bradyzoite-based Western blot tests under non-reducing conditions (Table 2). The ENV-Toulouse tachyzoite-based Western blot under non-reducing conditions showed high Sp (98.5%) but lower Se (91.0%) than the above-mentioned Western blots performed under similar conditions. The tachyzoite-based Western blot test carried out by SALUVET-Madrid under reducing conditions showed lower levels of Se but 100% Sp (Table 2). The in house ELISA showed 97.3% Se *versus* 94.6% Sp, while the INGEZIM BES 12.BES.K1 INGENASA showed 97.2% Se and 93.0% Sp. The PrioCHECK ELISA 2.0 test showed 100% Se and 98.8% Sp, and the ID Screen Besnoitia indirect IDVET yielded 97.2% Se and 100% Sp. The FLI-Wusterhausen IFAT showed 100% Se and 95.4% Sp, while the SALUVET-Madrid IFAT showed 90.0% Se and 86.1% Sp (Table 2). Relative to the gold standard 'Majority of test plus pre-test info', Se significantly decreased, while Sp increased or was unchanged (Table 2).

Table 2. Se and Sp relative to gold standard criteria before TG-ROC analysis (n = 240).

T: tachyzoite; B: bradyzoite; nr: under non-reducing conditions; r: under reducing conditions.

	Majority test		*Majority test plus pre-test info*	
	Se (95%CI)	Sp (95%CI)	Se (95%CI)	Sp (95%CI)
SALUVET-Madrid T nr WB	98.1 (95.6-100)	97.7 (95.1-100)	88.4 (82.7-94.1)	98.3 (95.9-100)
SALUVET-Madrid B nr WB	99.0 (97.3-100)	97.7 (95.1-100)	89.2 (83.8-94.8)	98.3 (95.9-100)
SALUVET-Madrid T r WB	88.8 (82.8-94.8)	100 (100-100)	79.8 (72.6-87.0)	100 (100-100)
SALUVET-Madrid B r WB	95.4 (91.5-99.3)	96.8 (93.8-99.9)	86.1 (80.0-92.2)	97.4 (94.4-100)
FLI-Wusterhausen T nr WB	100 (100-100)	97.7 (95.1-100)	90.2 (85.0-95.5)	98.3 (95.9-100)
FLI-Wusterhausen B nr WB	99.1 (97.3-100)	98.5 (96.3-100)	90.2 (85.0-95.5)	100 (100-100)
ENV-Toulouse T nr WB	91.0 (85.5-96.3)	98.5 (96.3-100)	81.3 (74.4-88.2)	98.3 (95.9-100)
SALUVET-Madrid in house ELISA	97.3 (94.2-100)	94.6 (90.7-98.5)	87.8 (82.0-93.6)	94.9 (90.9-98.9)
INGEZIM BES 2.BES.K1 INGENASA ELISA	97.2 (94.1-100)	93.0 (88.6-97.4)	88.3 (82.6-94.1)	93.2 (88.6-97.7)
ID Screen Besnoitia indirect IDVET ELISA	97.2 (94.2-100)	100 (100-100)	86.9 (80.9-92.9)	100 (100-100)
PrioCHECK ELISA 2.0	100 (100-100)	98.8 (93.8-99.9)	91.9 (87.0-96.7)	99.1 (97.4-100)
SALUVET-Madrid IFAT	90.0 (84.4-95.6)	86.1 (80.2-92.1)	82.1 (75.3-88.9)	86.3 (80.1-92.5)
FLI-Wusterhausen IFAT	100 (100-100)	95.4 (91.8-99.0)	91.9 (87.0-96.8)	97.4 (94.6-100)

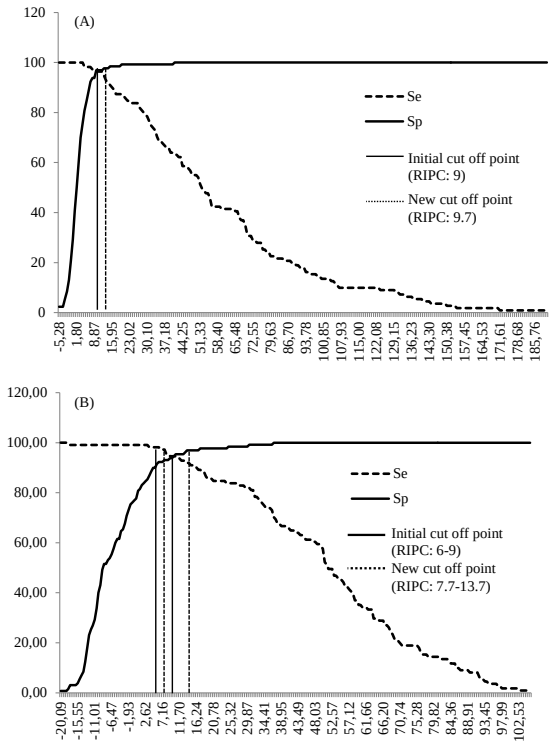
3.2. *TG-ROC analysis*

TG-ROC analysis were conducted to check the accuracy of the cut-offs of the ELISAs used in this study relative to the analysis based on the 'Majority of tests' (Fig. 1).

A higher cut-off (RIPC of 9.7) involved a slight increase in the Sp of the in house ELISA without a substantial loss of Se. The recalculated and more conservative cut-off of the INGEZIM BES 12.BES.K1 INGENASA test, with doubtful results when RIPC values were between 7.7 and 13.7, increased Sp to 97.6%, whereas Se decreased to 95.3%. For the ID Screen Besnoitia indirect IDVET test, the cut-off standardization by TG-ROC yielded a similar cut-off to the one currently suggested. TG-ROC analysis was not performed for the PrioCHECK ELISA 2.0, because this test showed almost perfect Se and Sp values.

Finally, acutely infected cattle as well as seronegative animals with tissue cysts were examined further (Table 3). All tests detected the only serum sample corresponding to the acute anasarca stage. The bradyzoite-based Western blot test under reducing conditions (carried out by SALUVET-Madrid), the bradyzoite and tachyzoite-based Western blot tests performed under non-reducing conditions (carried out by SALUVET-Madrid and FLI-Wusterhausen), the tachyzoite-based Western blot performed under non-reducing conditions (carried out by ENV-Toulouse), the INGEZIM BES 12.BES.K1 INGENASA ELISA and the FLI-Wusterhausen IFAT detected 30% (4/13) of the acutely infected animals in the febrile stage of the disease. Only the PrioCHECK ELISA 2.0 ELISA and the SALUVET-Madrid IFAT detected 38% (5/13) of these animals. Two of three chronically infected cattle showing tissue cysts that were previously considered seronegative were shown to be positive by tachyzoite- or bradyzoite- based Western blot tests under non-reducing conditions (carried out by SALUVET-Madrid and FLI-Wusterhausen) and both IFAT tests.

Fig. 1. TG-ROC analysis based on the gold standard 'Majority of tests' for in house ELISA (A) and INGEZIM BES 12.BES.K1 INGENASA (B).



3.3. *Test agreement (k-statistics)*

All tests showed almost perfect agreement (*k*= 0.8-0.9), with the exception of the pairwise comparisons that included the SALUVET-Madrid IFAT test, as this test showed the lowest Se values. The best *k* values (> 0.95) were obtained when comparing Western blot tests under non-reducing conditions carried out by SALUVET-Madrid and FLI-Wusterhausen, all ELISA tests and FLI-Wusterhausen IFAT.

K-values were calculated again using the adjusted cut-offs obtained by the TG-ROC analysis on the basis of the gold standard 'Majority of tests'. As expected from the fact that there was no significant adjustment of cut-offs, the increment of *k*-values was not substantial (Table 4).

3.4. *Cross-reactions*

Sixteen of 37 sera positive for *T. gondii* and *N. caninum* infections were positive by at least one of the techniques evaluated. SALUVET-Madrid IFAT showed higher number of cross-reactions (6/30), all with sera positive for *N. caninum* infection. Concerning ELISA tests, only INGEZIM BES 12.BES.K1 INGENASA showed cross-reactions to *T. gondii*-positive sera (n = 1). In house ELISA and INGEZIM BES 12.BES.K1 INGENASA showed three and two positive results, respectively, for *N. caninum*- positive sera. Concerning the Western blot, only SALUVET-Madrid tests based on tachyzoite and bradyzoite extracts (under non-reducing and reducing conditions, respectively) showed cross-reactions with one and three sera to *N. caninum*-positive sera, respectively. When adjusted cut-offs were used, the SALUVET-Madrid in house ELISA and INGEZIM BES 12.BES.K1 INGENASA test only yielded one positive result with *N. caninum*-positive serum.

Table 3. Detection of febrile, anasarca acute stages and seronegativity with tissue cysts sera samples.

T: tachyzoite; B: bradyzoite; nr: under non-reducing conditions; r: under reducing conditions.

Tests	Fever acute stage	Anasarca acute stage	Seronegative animals with tissue cysts
SALUVET-Madrid T nr WB	4/13	1/1	2/3
SALUVET-Madrid B nr WB	4/13	1/1	2/3
SALUVET-Madrid T r WB	3/13	1/1	1/3
SALUVET-Madrid B r WB	4/13	1/1	1/3
FLI-Wusterhausen T nr WB	4/13	1/1	2/3
FLI-Wusterhausen B nr WB	4/13	1/1	2/3
ENV-Toulouse T nr WB	4/13	1/1	1/3
SALUVET-Madrid in house ELISA	2/13	1/1	0/3
INGEZIM BES 2.BES.K1 INGENASA ELISA	4/13	1/1	1/3
ID Screen Besnoitia indirect IDVET ELISA	3/13	1/1	1/3
PrioCHECK ELISA 2.0	5/13	1/1	1/3
SALUVET-Madrid IFAT	5/13	1/1	2/3
FLI-Wusterhausen IFAT	4/13	1/1	2/3

3.5. Reproducibility

The CV was calculated for the PrioCHECK ELISA 2.0 test. The results obtained by all partners showed almost perfect agreement (k > 0.8); however, the best results were obtained by SALUVET-Madrid, FLI-Wusterhausen and IPB-Berne (k > 0.9). Only 29 samples of 241 showed CV values lower than 10%. 86 sera showed values between 10% and 20%, while the majority of sera showed values higher than 20% (52.2%). Twenty-three sera showed discrepancies in results among different laboratories (most with PP values near the cut-off point); however, these quantitative differences did not affect discrimination between seropositive and seronegative sera when the gold standard 'Majority of tests' was considered.

4. Discussion

The standardization of serological tests is crucial to broadening knowledge of the epidemiology of bovine besnoitiosis, a disease considered to be emergent in Europe. There is currently a lack of well-designed cross-sectional studies investigating the prevalence and incidence in endemic areas of Western Europe (EFSA, 2010). Therefore, the real impact of the disease remains unknown. A strategy combining diagnosis and control is urgently needed, especially because there are no effective drugs and vaccines, and this parasitic disease is spreading rapidly. In an outbreak of bovine besnoitiosis, almost an entire herd may become infected in less than three years (Fernández-García et al., 2010; Rostaher et al., 2010). Thus, standardization of serological tests is the first step towards disease control because it will make results from different countries comparable; thus, countries can adopt common diagnostics procedures for disease control.

Control measures should rely on the detection of infected cattle to avoid introducing *B. besnoiti* into a cattle population; however, affected countries and those at risk of acquiring the disease have not adopted common control strategies. A reliable diagnosis should include both clinical inspection and serological screening of cattle. It is important that during the acute stage of the disease, most clinical signs are non-specific for bovine besnoitiosis and could be related to a number of other infectious diseases. Therefore, a definitive clinical diagnosis in this phase of disease is not possible. Further, the majority of animals in an infected herd develop only subclinical infections. As shown by Fernández-García et al. (2010) in a study performed in central Spain, only 43.2% (154/358) of examined animals showed at least one clinical sign of bovine besnoitiosis, while 90.5% of the animals tested seropositive. Therefore, sensitive and specific diagnostic tests are needed to supplement the clinical assessment of cattle at risk. Although all tests available in Europe perform well during the chronic phase of the disease (Cortes et al., 2006a; Fernández-García et al., 2010; Schares et al., 2010), all show low sensitivity during both the acute febrile and anasarca stages, as detectable antibody levels have not yet developed. Additionally, a low percentage of chronically infected animals remain undiagnosed as a result of a low level of circulating antibodies. Furthermore, seronegative animals with tissue cysts have also been described (Fernández-García et al., 2010; Schares et al., 2010).

Table 4. Test agreement after TG-ROC analysis.

	k-values (95% CI)						
Test	1	2	3	4	5	6	7
1		**0.97 (0.94-1.00)**	0.87 (0.80-0.93)	0.89 (0.83-0.95)	**0.97 (0.93-1.00)**	0.94 (0.90-0.98)	0.86 (0.80-0.93)
2	**0.97 (0.94-1.00)**		0.86 (0.80-0.93)	0.90 (0.84-0.95)	**0.97 (0.95-1.00)**	0.95 (0.91-0.99)	0.86 (0.79-0.92)
3	0.87 (0.80-0.93)	0.86 (0.80-0.93)		0.90 (0.85-0.96)	0.86 (0.80-0.93)	0.87 (0.81-0.93)	0.86 (0.79-0.93)
4	0.89 (0.83-0.95)	0.90 (0.84-0.95)	0.90 (0.85-0.96)		0.89 (0.83-0.95)	0.91 (0.86-0.97)	0.84 (0.77-0.91)
5	**0.97 (0.93-1.00)**	**0.97 (0.95-1.00)**	0.86 (0.80-0.93)	0.89 (0.83-0.95)		0.96 (0.92-0.99)	0.88 (0.82-0.94)
6	0.94 (0.90-0.98)	0.95 (0.91-0.99)	0.87 (0.81-0.93)	0.91 (0.86-0.97)	0.96 (0.92-0.99)		0.87 (0.81-0.94)
7	0.86 (0.80-0.93)	0.86 (0.79-0.92)	0.86 (0.79-0.93)	0.84 (0.77-0.91)	0.88 (0.82-0.94)	0.87 (0.81-0.94)	
8	0.91 (0.86-0.97)	0.93 (0.89-0.98)	0.84 (0.77-0.91)	0.88 (0.82-0.94)	0.92 (0.88-0.97)	0.92 (0.87-0.98)	0.82 (0.75-0.90)
9	0.90 (0.85-0.96)	0.91 (0.86-0.96)	0.84 (0.76-0.91)	0.87 (0.80-0.93)	0.92 (0.87-0.97)	0.91 (0.86-0.97)	0.86 (0.79-0.93)
10	0.93 (0.75-0.89)	0.94 (0.90-0.98)	0.88 (0.81-0.94)	0.91 (0.81-0.94)	0.94 (0.90-0.98)	0.95 (0.91-0.99)	0.91 (0.86-0.97)
11	0.94 (0.90-0.98)	0.95 (0.90-0.99)	0.85 (0.78-0.92)	0.90 (0.84-0.95)	0.96 (0.92-0.99)	**0.97 (0.93-1.00)**	0.86 (0.80-0.93)
12	0.72 (0.63-0.81)	0.71 (0.63-0.80)	0.74 (0.66-0.83)	0.73 (0.64-0.82)	0.73 (0.64-0.81)	0.73 (0.65-0.82)	0.72 (0.79-0.92)
13	0.92 (0.90-0.98)	0.93 (0.90-0.99)	0.84 (0.77-0.91)	0.88 (0.82-0.94)	0.94 (0.90-0.98)	0.95 (0.91-0.99)	0.86 (0.80-0.93)

	8	9	10	11	12	13
1	0.91 (0.86-0.97)	0.90 (0.85-0.96)	0.93 (0.75-0.89)	0.94 (0.90-0.98)	0.72 (0.63-0.81)	0.92 (0.90-0.98)
2	0.93 (0.89-0.98)	0.91 (0.86-0.96)	0.94 (0.90-0.98)	0.95 (0.90-0.99)	0.71 (0.63-0.80)	0.93 (0.90-0.99)
3	0.84 (0.77-0.91)	0.84 (0.76-0.91)	0.88 (0.81-0.94)	0.85 (0.78-0.92)	0.74 (0.66-0.83)	0.84 (0.77-0.91)
4	0.88 (0.82-0.94)	0.87 (0.80-0.93)	0.91 (0.81-0.94)	0.90 (0.84-0.95)	0.73 (0.64-0.82)	0.88 (0.82-0.94)
5	0.92 (0.88-0.97)	0.92 (0.87-0.97)	0.94 (0.90-0.98)	0.96 (0.92-0.99)	0.73 (0.64-0.81)	0.94 (0.90-0.98)
6	0.92 (0.87-0.98)	0.91 (0.86-0.97)	0.95 (0.91-0.99)	**0.97 (0.93-1.00)**	0.73 (0.65-0.82)	0.95 (0.91-0.99)
7	0.82 (0.75-0.90)	0.86 (0.79-0.93)	0.91 (0.86-0.97)	0.86 (0.80-0.93)	0.72 (0.79-0.92)	0.86 (0.80-0.93)
8		0.89 (0.83-0.95)	0.90 (0.84-0.95)	0.92 (0.86-0.97)	0.68 (0.59-0.78)	0.90 (0.84-0.95)
9	0.89 (0.83-0.95)		0.92 (0.87-0.94)	0.91 (0.86-0.96)	0.71 (0.61-0.80)	0.90 (0.84-0.95)
10	0.90 (0.84-0.95)	0.92 (0.87-0.94)		0.93 (0.89-0.98)	0.75 (0.66-0.83)	0.92 (0.87-0.97)
11	0.92 (0.86-0.97)	0.91 (0.86-0.96)	0.93 (0.89-0.98)		0.73 (0.64-0.82)	**0.97 (0.95-1.00)**
12	0.68 (0.59-0.78)	0.71 (0.61-0.80)	0.75 (0.66-0.83)	0.73 (0.64-0.82)		0.72 (0.63-0.81)
13	0.90 (0.84-0.95)	0.90 (0.84-0.95)	0.92 (0.87-0.97)	**0.97 (0.95-1.00)**	0.72 (0.63-0.81)	

T, tachyzoite; B, bradyzoite; nr, under non-reducing conditions; r, under reducing condition; 1, SALUVET-Madrid T nr WB; 2, SALUVET-Madrid B nr WB; 3, SALUVET-Madrid T r WB; 4, SALUVET-Madrid B r WB; 5, FLI-Wusterhausen T nr WB; 6, FLI-Wusterhausen B nr WB; 7, ENV-Toulouse T nr WB; 8, SALUVET-Madrid in-house ELISA; 9, INGEZIM BES 12.BES.K1 INGENASA; 10, ELISA ID Screen Besnoitia indirect IDVET; 11, PrioCHECK ELISA 2.0; 12, SALUVET-Madrid IFAT; 13, FLI-Wusterhausen IFAT. The highest *k* values are shown in bold.

This study compares the serological tests available in Europe with a standard panel of sera to determine a gold standard technique for the diagnosis of bovine besnoitiosis.

Almost all the tests used showed a high level of agreement, indicating good performance by all techniques. When the 'Majority of tests' gold standard was considered, all tests showed higher Se and Sp values than when 'Majority of tests plus Pre-test info' was considered; this result is easily explained by the inclusion of sera from seronegative animals with clinical signs. All ELISAs performed well. The best-adjusted ELISAs were the ID Screen Besnoitia indirect IDVET tests and the PrioCHECK ELISA 2.0 (the second only when the result obtained by the majority of laboratories was considered). The SALUVET-Madrid in house ELISA and the INGEZIM BES 12.BES.K1 INGENASA test yielded similar Se and Sp results. These results were expected because the INGEZIM BES 12.BES.K1 INGENASA test is based on the test developed by the SALUVET group (Fernández-García et al., 2010). As expected, a further increase in agreement, Se and Sp for the SALUVET-Madrid in house ELISA and the INGEZIM BES 12.BES.K1 INGENASA ELISA test were obtained when the adjusted cut-offs from the TG-ROC analysis were applied; however, the non-significant improvement indicates an initial appropriate adjustment of all ELISAs.

In general terms, the PrioCHECK ELISA 2.0 performed well when the gold standard 'Majority of tests' was considered. This test was recently licensed in Germany and introduced to the European market. Nevertheless, CV values showed noticeable differences among participants performing the test. Thus, laboratories and manufacturers should carefully check all steps to ensure standard performance of the test. Given these results, reproducibility should be also tested for the other ELISAs included in the present study.

Western blot tests performed under non-reducing conditions and carried out by SALUVET-Madrid and FLI- Wusterhausen obtained the highest values of Se and Sp, regardless of the antigenic extract employed. Contrary to expectations, there were no significant differences in Se and Sp between tachyzoite- and bradyzoite-based Western blot tests in detection of acute cases of the disease or chronically infected animals, respectively. This similarity may have resulted because both parasite stages may share most of their surface proteins, and the bradyzoite-specific proteins may remain unexposed to the immune system as an evasion mechanism. Moreover, the SALUVET-Madrid tachyzoite-based Western blot test performed under reducing conditions showed 100% Sp and 88.8% Se. Although the seropositive criteria employed seemed highly restrictive, Se was not increased when other criteria were employed (data not shown). Tachyzoite-based Western blot tests performed under non-reducing conditions in ENV-Toulouse performed similarly to the SALUVET-Madrid tachyzoite-based Western blot under reducing conditions, with 90.9% and 98.5% Se and Sp, respectively. These results support the hypothesis that differences among Western blots are mainly related to the performance of the tests (e.g. protocols, reagents, antigen bands inspected). The lower Se value observed in ENV-Toulouse could also be explained by the more stringent criteria used to consider a serum as

positive. The FLI- Wusterhausen IFAT test performed better than IFAT SALUVET-Madrid. Differences may be attributed to the quality of reagents employed by both laboratories, as fixed tachyzoites are likely to be similar and cut-offs seem to be well adjusted (Fernández-García et al., 2009a; Schares et al., 2010). Indeed, if SALUVET-Madrid had also considered a higher cut-off point (1:200), this would have resulted in lower values of Se despite a substantial improvement in Sp (data not shown).

Considering the present limitations of serological tools, none of the techniques analysed in the study led to the detection of a significant number of samples representative of the acute febrile stage. Moreover, the number of samples included in the study is not high enough to study the performance of these tests for this purpose. Thus, experimental infections should be carried out in bovines, with careful monitoring of the infection and collection of serial blood samples. Unfortunately, several authors have failed to develop a reproducible experimental bovine model of besnoitiosis (Basson et al., 1970; Diesing et al., 1988). Secondly, almost all seronegative but chronically infected cattle with tissue cysts were positive by tachyzoite- and bradyzoite-based Western blot tests under non-reducing conditions and by SALUVET-Madrid and FLI-Wusterhausen IFAT. Moreover, the fact that a small proportion of chronically infected animals with tissue cysts are missed by serological tools may go unnoticed, as the diagnostic protocol should include both clinical inspection and serological analysis; however, the detection of cysts on conjunctival sclera is not always easy.

Considering cross-reactions, it is known that there are cross-reactive antigens among Sarcocystidae (Cortes et al., 2006a; Fernández-García et al., 2009a; Schares et al., 2010). In particular, some *Neospora*-positive animals may be responsible for false-positive results when investigating *B. besnoiti* infection (Schares et al., 2011a). This situation may be problematic, as bovine neosporosis is distributed worldwide, and high seroprevalence rates have been reported in Europe (Bartels et al., 2006); however, the obtained results suggest that cross-reactions were not significant.

On the basis of the results obtained in the present work and to establish a multi-national control programme to carry out prevalence and incidence studies, any of the tested ELISAs could be used to determine the initial serological status of a herd because of their easy performance and good diagnostic characteristics. Additionally, a Western blot test with both Se and Sp values near 100% should be recommended only to re-test animals with ambiguous results, cattle prior to entry to herds free of the disease and valuable animals prior to a selective culling.

Currently, the serological techniques standardized in this study do not overcome the limitations on detecting infected animals during the acute stage of the disease. Thus, the development of new tests based on early adhesion-invasion (either tachyzoite- or bradyzoite-stage-specific proteins) and PCR tests for detecting parasitemia during the early stages of *B. besnoiti* infection may be feasible options. Indeed, some authors (Cortes et al., 2007b; Schares et al., 2011b) have developed PCR and real-time-PCR tests for bovine skin biopsies that allow the detection of *B. besnoiti* even in samples that

were collected from seropositive but subclinically infected animals. Parasitemia has so far been reported only in animals that had recently become infected (Bigalke, 1968).

This comparison of serological tools has already been previously carried out for other infectious diseases affecting cattle, such as *N. caninum* (Von Blumröder et al., 2004; Wapenaar et al., 2007), Q fever (Horigan et al., 2011), *Babesia bigemina* (Singh et al., 2009) and bovine fasciolosis (Ibarra et al., 1998); these tests made possible prevalence studies and the establishment of common control plans. The success of this kind of comparative study evidences the need for further validation of future tests, such as the sensitive and specific recently published agglutination test developed by Waap et al. (2011).

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Objetivo 2. Investigación del origen de los resultados falsos-positivos en el ELISA para la detección de anticuerpos frente a la infección por *B. besnoiti.*

Las medidas de control disponibles para evitar la diseminación de la besnoitiosis bovina se basan en la detección de animales infectados (mediante un diagnóstico clínico y serológico) y en la aplicación de medidas de manejo adecuadas, lo que permite reducir la prevalencia en zonas endémicas y evitar la entrada de la enfermedad en zonas libres a través de la compra de animales infectados. En relación a las técnicas de diagnóstico serológico, es necesario mejorar su Esp, ya que las actuales pruebas ELISA, que se emplean de forma rutinaria en el diagnóstico de la infección, pueden dar lugar a un elevado número de resultados falsos-positivos que comprometen la eficacia de los planes de control. En este trabajo, se ha estudiado el origen de las reacciones cruzadas serológicas entre antígenos del taquizoíto de *B. besnoiti,* y anticuerpos específicos anti-*N. caninum* y/o anti-*Sarcocystis* spp., ya que son parásitos filogenéticamente cercanos y sus infecciones son muy prevalentes en el ganado bovino a nivel mundial. En el panel de sueros se han incluido las siguientes categorías: 75 sueros seronegativos (categoría 1) y 66 sueros seropositivos a *B. besnoiti* (categoría 2), 96 sueros con un resultado falso-positivo en el ELISA *in house* de *B. besnoiti* "BbSALUVET ELISA 1.0" (categoría 3), 42 sueros seropositivos a *Sarcocystis* spp. (categoría 4) y 36 sueros seropositivos a *N. caninum* (categoría 5). En este trabajo se ha considerado el Western blot en condiciones no reductoras como la técnica de referencia para clasificar los sueros como verdaderos seronegativos o seropositivos. En todos los sueros incluidos en el estudio se ha confirmado la presencia de anticuerpos específicos anti-*Sarcocystis* spp. y anti-*N. caninum* mediante Western blot y se ha determinado el nivel de anticuerpos mediante IFAT y ELISA, respectivamente. Para la realización de este trabajo, en primer lugar se ha descrito el criterio de seropositividad en el Western blot de cistozoítos de *Sarcocystis* spp. para la detección de animales infectados y se ha determinado que el 100% de los animales infectados, reconocen de forma intensa un área antigénica de aproximadamente 18-20

kDa. En relación a los resultados, cabe destacar que el número de sueros seropositivos a *N. caninum* y *Sarcocystis* spp. ha sido significativamente mayor en la categoría 3 (bovinos con un resultado de ELISA falso-positivo) (74%), seguido por la categoría 5 (bovinos seropositivos a *N. caninum*) (52,8%). Por el contrario, los sueros incluidos en el resto de categorías, casi no ha mostrado resultados seropositivos a *N. caninum* y/o *Sarcocystis* spp. Adicionalmente, se ha observado que los sueros incluidos en la categorías 3, mostraron niveles de anticuerpos específicos anti-*Sarcocystis* spp. y anti-*N. caninum* significativamente más elevados ($p < 0,05$, *t* de Student). Este trabajo ha puesto de manifiesto que los resultados de BbSALUVET ELISA 1.0 falsos-positivos están asociados no sólo a la presencia de anticuerpos específicos anti-*Sarcocystis* spp. y anti-*N. caninum* (χ^2: 78,36; $p < 0,0001$; OR = 34,65; CI: 13,6 - 88,5), sino también a un elevado nivel de anticuerpos frente a ambas. Además ha puesto de manifiesto la importancia de incluir un alto número de sueros procedentes de animales seropositivos a *Sarcocystis* spp. y *N. caninum*, y con niveles elevados de anticuerpos frente a ambos parásitos, para la validación y estandarización de las pruebas de diagnóstico serológico.

Anti-*Neospora caninum* and anti-*Sarcocystis* spp. specific antibodies cross-react with *Besnoitia besnoiti* and influence the serological diagnosis of bovine besnoitiosis

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Abstract

Bovine besnoitiosis control remains a challenge because the disease continues to spread and control relies solely on accurate diagnosis coupled to management measures. However, recent studies have reported that routinely used ELISAs may raise a high number of false-positive results. Herein, cross-reactions between *Besnoitia besnoiti* antigens and anti-*Neospora caninum* and/or anti-*Sarcocystis* spp.-specific antibodies were studied in an in house ELISA since *N. caninum* and *Sarcocystis* spp. are closely related parasites, and both infections are highly prevalent in cattle worldwide. The serum panel was composed of the following categories: sera from *B. besnoiti*-seronegative (n = 75) and -seropositive cattle (n = 66), *B. besnoiti*-based-ELISA false-positive reactors (n = 96) together with *N. caninum* (n = 36) and *Sarcocystis* spp. (n = 42) -seropositive reference cattle sera. *B. besnoiti* tachyzoite-based Western blot (WB) results classified animals as seropositive or seronegative. Sera were analyzed for the detection of anti-*N. caninum* by WB and ELISA and anti-*Sarcocystis* spp.-specific antibodies by WB and IFAT. Those samples recognizing a *Sarcocystis* spp. 18-20 kDa antigenic region and *N. caninum* 17-18 kDa immunodominant antigen were considered to be *Sarcocystis* spp. and *N. caninum* seropositive, respectively. The category of *B. besnoiti* based-ELISA false-positive reactors showed the highest number of sera with specific anti-*Sarcocystis* spp. and anti-*N. caninum* antibodies (74%; 71/96), followed by the *N. caninum*-seropositive cattle category (52.8%; 19/36). In contrast, few *B. besnoiti*-seronegative and -seropositive cattle showed antibodies against *Sarcocystis* spp. and *N. caninum* (10.7%; 8/75 and 1.5%; 1/66), respectively). This study revealed that *B. besnoiti* false-positive ELISA results were associated not only with the presence of anti-*N. caninum* and anti-*Sarcocystis* spp. antibodies (χ^2: 78.36; $p < 0.0001$; OR: 34.6; CI: 14-88) but also with high antibody levels against them using ELISA and IFAT tests, respectively ($p < 0.05$; *t*-test). These results may explain why only some animals seropositive to *Sarcocystis* spp. and/or *N. caninum* are *Besnoitia* false-positive reactors. Therefore, sera meeting these requirements should be included in future validations of serological tests for bovine besnoitiosis.

Keywords: *Besnoitia besnoiti*; *Neospora caninum; Sarcocystis* spp.; Cattle; Serological diagnosis; Cross-reactions.

1. Introduction

Besnoitia besnoiti is a cyst-forming apicomplexan parasite that belongs to the genus Besnoitia, family Sarcocystidae and subfamily Toxoplasmatinae. It is the causative agent of bovine besnoitiosis, a chronic and a debilitating disease responsible for severe economic losses, mainly due to poor body condition, sterility in bulls and eventual death (reviewed by Álvarez-García et al., 2014b). In Western Europe, there is concern over the spread of bovine besnoitiosis and recent epidemiological studies have revealed high seroprevalence rates in areas where the disease is endemic (Liénard et al., 2011; Álvarez-García et al., 2014a; Gutiérrez-Expósito et al., 2014).

At present, control relies on accurate diagnosis coupled to management measures as no effective drugs or vaccines are available. For an accurate diagnosis a combination of clinical inspection and serology is necessary in order to detect both acute and chronically infected animals with or without visible tissue cysts (subclinically infected cattle) (Frey et al., 2013b; García-Lunar et al., 2013a). For this purpose, a number of serological methods including IFAT, ELISA, Western blot (WB) and direct agglutination tests have been developed (Cortes et al., 2006a; Fernández-García et al., 2009a, 2010; Schares et al., 2010, 2013; Waap et al., 2011). However, previously validated ELISA tests that are frequently employed in diagnosis and epidemiological studies have shown false-positive results that may significantly influence prevalence studies and control (Schares et al., 2010; Nasir et al., 2012; Gazzonis et al., 2014). Moreover, Uzêda et al. (2014) also reported the presence of *Besnoitia* false-positive results by IFAT. Therefore, the WB test is currently recommended as a confirmatory assay (García-Lunar et al., 2013a).

False-positive reactions have been suggested to be due to cross-reactions with related apicomplexan parasites (Nasir et al., 2012; Gazzonis et al., 2014). Indeed, previous studies have identified shared antigens among members of the family Sarcocystidae (*Neospora caninum, Toxoplasma gondii* and *B. besnoiti*) (Zhang et al., 2011; García-Lunar et al., 2013b). In particular, *N. caninum* and *Sarcocystis* spp. are closely related parasites, and both infections are highly prevalent in cattle worldwide (Dubey et al., 1989; reviewed by Dubey, 2003). Over 90% of adult cattle have been reported to be infected with *S. cruzi* in many countries (Moré et al., 2008). However, serological cross-reactions between *Sarcocystis* spp. that affect cattle (*S. cruzi*, S. hirsuta, S. *hominis* and *S. rommeli*) (Dubey and Lindsay, 2006; Dubey et al., 2015) and *B. besnoiti* have never been studied. Shkap et al. (2002) determined that at low IFAT dilutions, anti-*N. caninum* sera reacted with *B. besnoiti* antigens in some individual samples. This finding may have important implications for the diagnosis as Bartels et al. (2006) reported high seroprevalence rates of *N. caninum* infection in several European countries.

The aim of the present study was to investigate the origin of *B. besnoiti* false-positive ELISA results using an appropriate sera panel. For this purpose, the association between *B. besnoiti* false-positive ELISA results obtained with an in house ELISA and the presence and level of anti-*Sarcocystis* spp. and/or *N. caninum*-specific antibodies were studied. We considered WB as a reference

test for *Sarcocystis* spp. infection diagnosis. Thus, we firstly determined the pattern of *Sarcocystis* spp. cystozoites antigen recognition. The presence and level of anti-*Sarcocystis* spp. and/or -*N. caninum* antibodies were then investigated.

2. Material and methods

2.1. Experimental design and serum samples

A total of 315 sera samples were included in the present study and were classified into five different groups (1, 2, 3, 4 and 5). Groups 1, 2 and 3 were established based on the results obtained with a *B. besnoiti* in house ELISA based on a soluble tachyzoite extract (Fernández-García et al., 2010) and a *B. besnoiti* tachyzoite-based WB performed under non-reducing conditions (García-Lunar et al., 2013a). For both assays, BbSpain-1 isolate was employed (Fernández-García et al., 2009b). WB was considered as gold standard (García-Lunar et al., 2013a). Group 1 and Group 2 were composed of sera from *B. besnoiti*-seronegative and -seropositive animals, respectively, according to both ELISA and WB analysis. Group 3 comprised sera from *B. besnoiti*-seronegative animals by WB with a false-positive ELISA result. Groups 4 and 5 corresponded to reference sera representative of *Sarcocystis* spp. and *N. caninum* infections, respectively.

In all groups, the presence of anti-*Sarcocystis* spp. antibodies was determined using WB and the anti-*Sarcocystis* spp. specific antibody level was determined by IFAT as described in sections 2.3 and 2.4, respectively. The presence of anti-*N. caninum* antibodies was determined using WB, whereas ELISA was employed for determining the anti-*N. caninum* specific antibody level (Álvarez-García et al., 2002, 2003).

Group 1: Sera from B. besnoiti-seronegative cattle

The sera from 75 cows and heifers from dairy and beef herds located in central Spain (n=20) (Fernández-García et al., 2010), Italy (n=18) (Gazzonis et al., 2014), Argentina (n=20) and Mexico (n=17) were included in the study. A total of 56 serum samples came from herds with no history of bovine besnoitiosis, and the 75 animals showed any compatible sign of besnoitiosis. All sera were seronegative for *B. besnoiti* infection by both WB and ELISA.

Group 2: Sera from B. besnoiti-seropositive cattle

Sera came from cows and heifers from beef herds with a previous history of bovine besnoitiosis. No data regarding clinical signs were available for any of the animals sampled. However, all sera were seropositive for *B. besnoiti* infection by both WB and ELISA.

Group 3: Sera from B. besnoiti-seronegative cattle with a false-positive ELISA result

A total of 96 sera from adult dairy and beef cattle from Argentina (n=6) and Italy (n=22) (Gazzonis et al., 2014) and from dairy cattle from Mexico (n=16) and Spain (n=52) were included in the study. No animals showed any compatible sign of besnoitiosis, and the animals came from herds with no history of bovine besnoitiosis. All sera were positive for *B. besnoiti* infection by ELISA but negative by WB.

Group 4: Sera from S. cruzi-seropositive cattle

Sera from 42 Argentinean heifers and cows naturally infected with *S. cruzi* that showed microscopic thin-walled tissue cysts in myocardium and with IFAT titers ≥ 1:100 (Moré et al., 2008) were included in the study. All sera were seronegative to *B. besnoiti* by both WB and ELISA and seronegative to *N. caninum* by both WB and ELISA (Álvarez-García et al., 2002, 2003). All samples were employed to describe the pattern of *Sarcocystis* spp. cystozoite antigens by WB.

Group 5: Sera from N. caninum-seropositive cattle

Sera from 36 dairy cattle present in a herd with a history of *N. caninum*-associated abortions were analyzed. The herd comprised 200 cows and had an intra-herd seroprevalence of 85% and a 9.2% annual abortion rate. All samples were seronegative for *B. besnoiti* by both WB and ELISA and seropositive for *N. caninum* by ELISA and WB.

2.2. Parasites

Sarcocystis spp. cystozoites were obtained from naturally infected bovine hearts and were purified according to a previously described procedure (Moré et al., 2011). Briefly, 100 g of minced myocardium were mixed with 400 ml of digestion solution (2.5% pepsin, 1% HCl) and were placed in a magnetic stirrer for 20 min at 37°C. The suspension was filtered through 300, 150, and 53 µm sieves into 50 ml centrifuge tubes and centrifuged at 500×g for 5 min and it was centrifuged at 500×g for 5 min. The supernatant was removed and the pellet was re-suspended in 30 ml PBS, 13.5 ml of isotonic Percoll® (GE Healthcare) and 1.5 ml saline solution (1.5M NaCl) and centrifuged (4,000 ×g, 10 min) (Pertoft et al., 1980). Supernatants and the upper layer of the sediment were discarded; the pellet was washed three times with PBS. Pellets with zoites were frozen at -80ºC until use for WB or resuspended in PBS to a final concentration of approximately 5×105 cystozoites per ml and formalin-fixed for their use in IFAT (Moré et al., 2008; Fernández-García et al., 2009a). Tachyzoites from the Nc-1 isolate of *N. caninum* (Dubey et al., 1988) were grown in a Marc-145 cell monolayer with DMEM (Thermo Fisher Scientific) supplemented with 2% fetal calf serum (Thermo Fisher Scientific) and were purified following a previously described procedure (Pérez-Zaballos et al., 2005). Tachyzoites were pelleted by centrifugation at 1,350 xg 15 min and stored at -80ºC until used for ELISA and WB.

2.3. SDS-PAGE and WB

A total of 2×10^7 tachyzoites under reducing conditions and 10^7 cystozoites under non-reducing conditions were employed for *N. caninum* and *Sarcocystis* spp. electrophoresis, respectively. Membrane coating and WB were performed following a previously described method (Álvarez-García et al., 2002) in a 15% polyacrylamide gel. Images from WB membranes were obtained using a GS-800 Scanner (Bio-Rad Laboratories, CA, USA) and were analyzed with Quantity One quantification software v. 4.0 (Bio-Rad Laboratories, CA, USA).

2.4. Sarcocystis spp. IFAT

Sera were analyzed by *Sarcocystis* spp. cystozoite IFAT in double serial dilutions

starting at 1:100 following a previously described method (Moré et al., 2008; Fernández-García et al., 2009a). Peripheral but not apical fluorescence was considered specific.

2.5. N. caninum in house ELISA

ELISA was performed following a previously described procedure (Álvarez-García et al., 2003). The optical density (OD) of each serum sample was converted into a relative index percent (RIPC) using the following formula: RIPC = [(OD405 sample - OD405 negative control) / (OD405 positive control - OD405 negative control)] × 100. Samples with RIPC ≤ 6.2 were considered negative; samples with RIPC = 6.2-8.2 were considered doubtful, and samples with RIPC ≥ 8.2 were considered positive.

2.6. Analysis of data

To determine the pattern of recognition of *Sarcocystis* spp. cystozoite antigens by WB, molecular weights of antigens were compared with the molecular weight marker and were calculated for each sample analyzed using Quantity One Software (Bio-Rad). The frequency of recognition was expressed as the percentage of animals detecting each antigen. An antigen was considered to be an immunodominant antigen (IDA) when it was recognized by more than 50% of samples analyzed.

The association between *B. besnoiti* ELISA false-positive results and the presence of anti-*N. caninum* and -*Sarcocystis* spp. antibodies by WB was estimated by using Chi-square test and by estimating the odds ratio value (OR). A Student's *t*-test was used to compare differences in the anti-*Sarcocystis* spp. or -*N. caninum* antibody levels estimated by ELISA and IFAT, respectively, between sera with *B. besnoiti* ELISA false-positive results and sera with no false-positive results. For this purpose, *Sarcocystis* spp. IFAT titers were expressed as $\log_2$ of the reciprocal of the last serum dilution that completely recognized the surface of the cystozoite. P values < 0.05 were considered statistically significant. Test agreement (expressed as Kappa-values) was calculated using WinEpiscope 2.0 (Thrusfield et al., 2001).

3. Results and discussion

Given the re-emergence of bovine besnoitiosis in Europe and the high prevalence rates recently reported in endemic areas, the implementation of control measures is urgently needed (reviewed by Álvarez-García et al., 2014c). In this scenario, serological diagnosis is crucial for detecting subclinical infected animals that may remain parasite carriers (reviewed by Álvarez-García et al., 2014c). However, recent studies have reported that routinely used commercial and in house ELISAs may produce between 18% and 30% false-positive results (Nasir et al., 2012; Gazzonis et al., 2014). Moreover, *Besnoitia*-positive IFAT results did not yield conclusive results by WB when Brazilian cattle were tested (Uzêda et al., 2014).

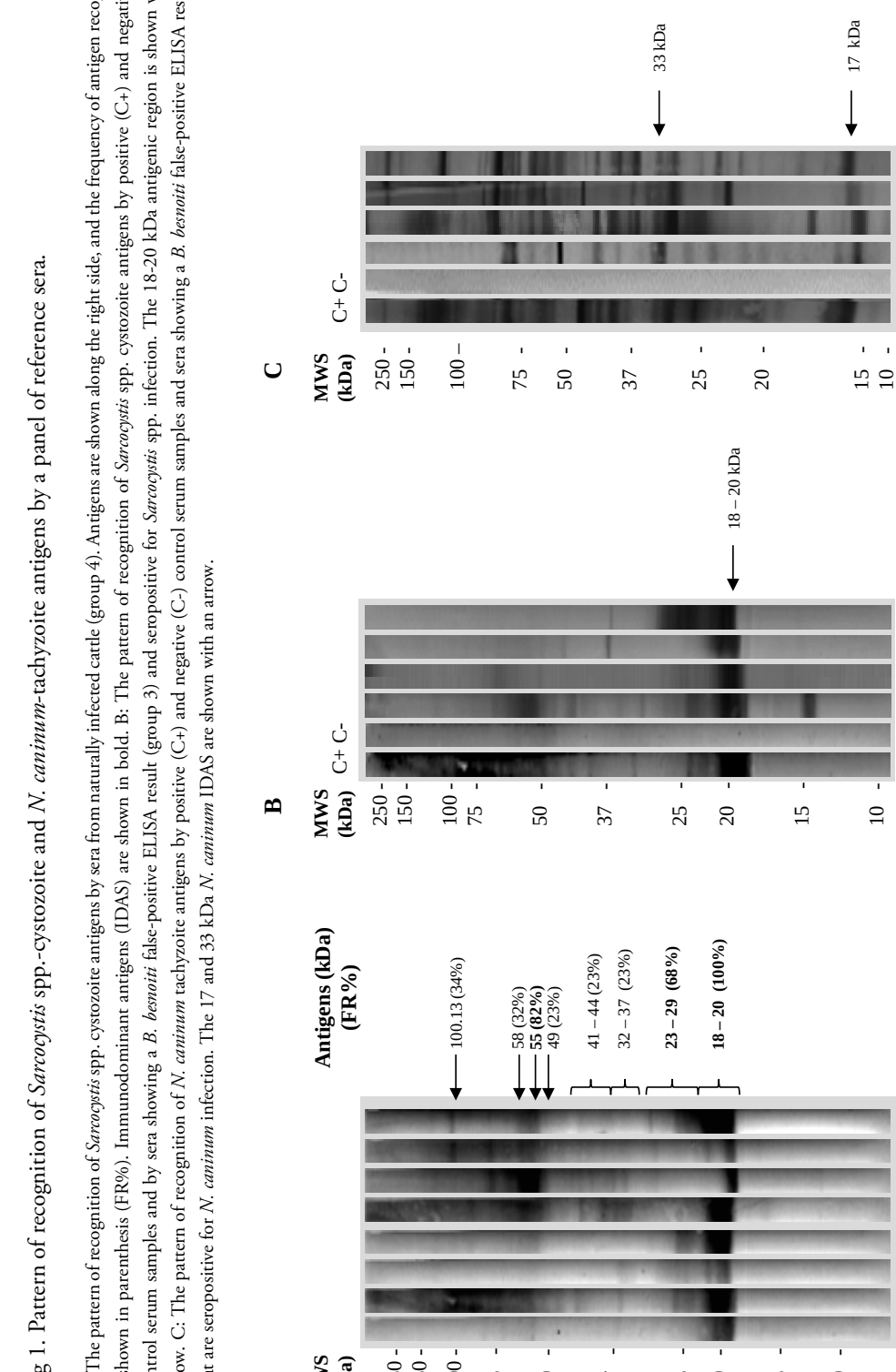
Thus, in the present study, the origin of false seropositive reactions in an in house ELISA was investigated for the first time. An appropriate panel of sera was employed and was composed of three categories that represented *B. besnoiti*-seronegative cattle with either an ELISA-negative or -positive result (false-positive reactors) and seropositive cattle. Since *N. caninum* and

Sarcocystis spp. infections are highly prevalent in cattle worldwide, we included two additional categories representative of seropositive cattle to both closely related protozoa. On the other hand, cross-reactions with *T. gondii* were not studied herein since in previous studies of development and validation of *B. besnoiti* serological assays cross-reactions between anti-*T. gondii* specific antibodies and *B. besnoiti* antigens were hardly observed (Fernández-García et al., 2010; Schares et al., 2011a, 2013; García-Lunar et al., 2013a). In contrast, optical density values close to the cut off points were commonly observed in *B. besnoiti* ELISAs with sera positive to *N. caninum* infection (Schares et al., 2010, 2013; García-Lunar et al., 2013a). Moreover, there are a few seroprevalence studies of *T. gondii* in cattle and the relevance of this parasitic infection in cattle remains to be further clarified. To classify sera as *Sarcocystis* spp.-seropositive or -seronegative in our study, WB was used as the reference technique as in previous studies with other closely related protozoa (Álvarez-García et al., 2002; García-Lunar et al., 2013a; Howe et al., 2014), whereas IFAT was employed for determining specific antibody levels.

Among a total of 26 *S. cruzi* cystozoite antigens recognized, 8 antigens were intensively recognized by reference sera from *S. cruzi* naturally infected cattle, which were located in 3 main antigenic regions: 55 kDa, 23-29 kDa (composed of 28.9, 27.2, 25.5, 23.9 kDa bands) and 18-20 kDa (composed of 20.8, 19.3, 18.4 kDa bands) (Fig. 1A). A serum was considered to be *Sarcocystis* spp. seropositive when at least the 18-20 kDa antigenic area was intensively recognized because it was detected in 100% of the infected animals (Fig. 1). A previous study carried out by Granstrom et al. (1990) described the immunodominant antigens of *S. cruzi* cystozoites and 20 bands, ranging from approximately 22 to 250 kDa, were consistently detected. However, these results are hardly comparable because 10% acrylamide gels were employed for the assays and antigens below 22 kDa were not visualized. Whether the antigens described in the present study are shared antigens among other species of *Sarcocystis* needs future clarification.

It is unclear whether WB or IFAT should be considered the gold standard for the detection of *Sarcocystis* spp.-specific antibodies. There have been scant studies on the serological diagnosis of *Sarcocystis* spp. infection in cattle. Moré et al. (2008) have described IFAT at low dilutions to be a suitable method for diagnosing *S. cruzi*-infected cattle. Cross-reactivity at low IFAT titers, however, has been widely described among other Sarcocystidae parasites (Shkap et al., 2002; Schares et al., 2010). In this study, a better agreement between IFAT and WB was obtained with a cut-off titer of 1:200 (77% sensitivity (Se); 79% specificity (Sp); k = 0.5) compared with a cut off titer of 1:100 (98% Se; 37.7% Sp; k = 0.4)". The use of *Sarcocystis* spp. cystozoite based WB as the gold standard in this work may have resulted in a substantial loss of Se. However, this approach as well as the restrictive WB seropositive criterion employed here may have resulted in high Sp. Unfortunately, due to the low number of reference sera from *S. cruzi* seropositive and seronegative cattle, the Se and Sp values of these tests could not be determined. Similarly, a moderate agreement between IFAT, WB and a modified WB were observed by Duarte et al.

Fig 1. Pattern of recognition of *Sarcocystis* spp.-cystozoite and *N. caninum*-tachyzoite antigens by a panel of reference sera.



A: The pattern of recognition of *Sarcocystis* spp. cystozoite antigens by sera from naturally infected cattle (group 4). Antigens are shown along the right side, and the frequency of antigen recognition is shown in parenthesis (FR%). Immunodominant antigens (IDAS) are shown in bold. B: The pattern of recognition of *Sarcocystis* spp. cystozoite antigens by positive (C+) and negative (C-) control serum samples and by sera showing a *B. besnoiti* false-positive ELISA result (group 3) and seropositive for *Sarcocystis* spp. infection. The 18-20 kDa antigenic region is shown with an arrow. C: The pattern of recognition of *N. caninum* tachyzoite antigens by positive (C+) and negative (C-) control serum samples and sera showing a *B. besnoiti* false-positive ELISA result and that are seropositive for *N. caninum* infection. The 17 and 33 kDa *N. caninum* IDAS are shown with an arrow.

Table 1. Detection of anti-*Sarcocystis* spp. and/or anti-*N. caninum* antibodies by WB.

Panel of reference sera	Nº of sera	Seropositive to *Sarcocystis* spp. (%)	Seropositive to *N. caninum* (%)	Seropositive to *N. caninum* and *Sarcocystis* spp. (%)	Seronegative to *N. caninum* and *Sarcocystis* spp. (%)
1. Sera from *B. besnoiti*-seronegative cattle	75	20 (26.7%)	4 (5.3%)	8 (10.7%)	43 (57.3%)
2. Sera from *B. besnoiti*-seropositive cattle	66	26 (39.4)	1 (1.5%)	1 (1.5%)	38 (57.6%)
3. Sera from *B. besnoiti*-seronegative cattle with a false-positive ELISA result	96	12 (12.5%)	7 (7.3%)	71 (74%)	6 (6.2%)
4. Sera from *S. cruzi*-seropositive cattle	42	42 (100%)	0	0	0
5. Sera from *N. caninum*-seropositive cattle	36	0	17 (47.2%)	19 (52.8%)	0
Total	315	100 (31.7%)	28 (8.9%)	99 (31.4%)	88 (27.9%)

(2003) for the diagnosis of *S. neurona* infection in equids. In this study performed by Duarte et al. (2003), the results of the operating characteristic (ROC) analysis determined that the overall accuracy of IFAT was higher than that of the WBs analyzed. In this sense, the existence of cross-reactivity with other species of *Sarcocystis* using WB has been described and its Sp depends on a careful interpretation of immunoreactive bands and/or modification of assay conditions (Arias et al., 2012).

Specific antibodies against *Sarcocystis* spp. and *N. caninum* were found in all categories examined (Fig. 1B-C, Table 1). However, 31.7% of the analyzed samples showed anti-*Sarcocystis* spp.-specific antibodies, whereas only 8.9% were seropositive for *N. caninum*. Notably, the category of *B. besnoiti* ELISA false-positive reactors showed the highest number of sera with specific antibodies directed against both *Sarcocystis* spp. and *N. caninum* (74%), followed by the *N. caninum*-seropositive animal category (52.8%) (Table 1). In contrast, few *B. besnoiti*-seronegative and -seropositive animals (categories 1 and 2, respectively) showed specific antibodies to both *N. caninum* and *Sarcocystis* spp. (10.7% and 1.5%, respectively)

The existence of *B. besnoiti* false-positive ELISA results was significantly associated with either *N. caninum* and/or *Sarcocystis* spp. seropositivity (93.8%; 90/96). The highest association was found between *Besnoitia* false-positive ELISA results and seropositive results to both *Sarcocystis* spp. and *N. caninum* infections ($\chi 2$: 78.36; $p < 0.0001$; OR = 34.65; 95% CI: 13.6-88.5). There was also a significant association with the presence of anti-*Sarcocystis* spp. antibodies, regardless of the *N. caninum* result ($\chi 2$: 29.90; $p < 0.0001$; OR = 5.56; 95% CI: 2.9-10.6). However, seropositivity to *Sarcocystis* spp. only was not associated with a *B. besnoiti* false-positive

ELISA result. (χ2: 0.91; p = 0.34; OR = 1.86; 95% CI: 0.67-5.2). This finding may be related to the restrictive WB seropositive criterion employed, resulting in a substantial loss of Se as mentioned above (Duarte et al., 2003). Regarding *N. caninum* infection, *B. besnoiti* false-positive ELISA results were also associated with seropositive *N. caninum* results, regardless of *Sarcocystis* spp. results (χ2: 93.72; p <0.0001; OR = 15.03; 95% CI: 8.2-27.5). Moreover, only *N. caninum* seropositive results were also associated with *B. besnoiti* false-positive ELISA results (χ2: 5.35; p = 0.02; OR = 4.56; 95% CI: 1.4-15). This result is in accordance with the findings of Schares et al. (2011a), who previously observed the presence of ELISA cross-reactions between *B. besnoiti* and anti-*N. caninum* antibodies while evaluating the Sp of a commercial ELISA test for the diagnosis of bovine besnoitiosis.

Table 2. Distribution of anti-*Sarcocystis* spp. and anti-*N. caninum* specific antibodies according to IFAT titers and ELISA relative index percent (RIPC) values, respectively.

[a] Sera samples showing discordant results between *Sarcocystis* spp. cystozoite based IFAT and WB under non-reducing conditions were not included in the statistical analysis.
[b] Sera samples showing doubtful ELISA results (≥ 6.2 ≤ 8.2) and sera showing discordant results between *N. caninum*-tachyzoite-based-ELISA and WB were not included in the statistical analysis.

	Sarcocystis spp. IFAT titres (%)					Total
Panel of reference sera [a]	< 1:100	1:100	1:200	1:400	1:800	
1. Sera from *B. besnoiti*-seronegative cattle	24 (46.2%)	7 (13.5%)	15 (28.8%)	6 (11.5%)	0	52
2. Sera from *B. besnoiti*-seropositive cattle	0	5 (20%)	16 (64%)	4 (16%)	0	25
3. Sera from *B. besnoiti*-seronegative cattle with a false-positive ELISA result	5 (5.7%)	15 (17.2%)	41 (47.1%)	24 (27.6%)	2 (2.3%)	87
4. Sera from *S. cruzi*-seropositive cattle	0	14 (33.3%)	24 (57.1%)	4 (9.5%)	0	42
5. Sera from *N. caninum*-seropositive cattle	14 (42.4%)	2 (6.1%)	14 (42.4%)	3 (9.1%)	0	33

	N. caninum ELISA RIPCs (%)					Total
Panel of reference sera [b]	< 6.2	> 8.2 ≤ 20	> 20 ≤ 40	> 40 ≤ 100	> 100	
1. Sera from *B. besnoiti*-seronegative cattle	60 (83.3%)	4 (5.6%)	3 (4.2%)	5 (6.9%)	0	72
2. Sera from *B. besnoiti*-seropositive cattle	61 (100%)	0	0	0	0	61
3. Sera from *B. besnoiti*-seronegative cattle with a false-positive ELISA result	7 (8.3%)	8 (9.5%)	23 (27.4%)	41 (48.8%)	5 (6%)	84
4. Sera from *S. cruzi*-seropositive cattle	42 (100%)	0	0	0	0	42
5. Sera from *N. caninum*-seropositive cattle	0	5 (13.9%)	14 (38.9%)	15 (41.7%)	2 (5.6%)	36

Specific antibody levels against *Sarcocystis* spp. and *N. caninum* were also measured by IFAT and ELISA, respectively (Table 2). Sera showing discordant results between serological techniques (IFAT and WB for *Sarcocystis* spp., and ELISA and WB for *N. caninum*) were not included in the study and were more numerous for *Sarcocystis* spp. infection diagnosis (n=76). On the other hand, only 20 samples showed discordant results for *N. caninum* infection. In general terms, *Sarcocystis* spp.-seropositive samples showed low IFAT antibody levels because approximately 90% sera showed IFAT titers equal to or lower than 1:200 (groups 1, 2, 4 and 5). Interestingly, those sera with a *B. besnoiti* false-positive ELISA result (group 3) showed higher antibody levels (more than 27% of the samples had *Sarcocystis* spp. IFAT titers equal or higher than 1:400). Our results contrast with those reported by Moré et al. (2008), where most Argentine cattle showed anti-*S. cruzi* antibody levels equal to or higher than 1:200. However, differences in the performance of the assays may explain these discrepancies. Regarding *N. caninum* infection, almost 50% of sera showing a *B. besnoiti* false-positive ELISA result had RIPC values higher than 40%, whereas between 80 and 100% of sera from groups 1, 2 and 4 proved to be seronegative. As expected, significantly higher specific antibody levels against *Sarcocystis* spp. and *N. caninum* corresponded to sera from group 3 (*B. besnoiti* seronegative animals with a positive ELISA result) ($p < 0.05$ t-test). These results showed that sera with high antibody levels anti-*N. caninum* and anti-*Sarcocystis* spp. are more likely to show *B. besnoiti* false-positive ELISA results. Our results agree with those reported in the study carried out by Shkap et al. (2002), in which cross-reactivity at low IFAT dilutions (1:16 and 1:64) was found only between two sera showing high anti-*N. caninum* antibody levels (1:3,200) and *B. besnoiti* antigen. Several *B. besnoiti* antigens responsible for these cross-reactions have recently been identified by 2-DE SDS-PAGE. Up to 25 *B. besnoiti*-cross-reactive antigens were recognized by a pool of sera from *N. caninum*-infected cattle and some of them were identified to be highly conserved enzymes involved in metabolism, such as heat shock protein 60 and 90, fructose 1-6 bisphosphatase aldolase, enolase and actin (García-Lunar et al., 2013b). When Schares et al. (2013) removed cross-reacting antigens with *N. caninum* during the development of an enriched membrane extract for ELISA, this new assay showed higher Se and Sp values using a serum panel that included 20 sera with a *B. besnoiti* false-positive and doubtful ELISA result (Nasir et al., 2012). In the closely related protozoa *T. gondii* and *N. caninum*, several antigens located on the surface of tachyzoites also improved the Se and Sp of the serological assays (Schares et al., 1999b; reviewed by Montoya and Liesenfeld, 2004; Dubey and Schares, 2006; Petersen and Liesenfeld, 2007).

4. Conclusion

In conclusion, this study revealed that sera with high antibody levels anti-*N. caninum* and anti-*Sarcocystis* spp. are more likely to show *B. besnoiti* false-positive in house ELISA results. This finding explains why only some animals co-infected with either *Sarcocystis* spp. and/or *N. caninum* may be *Besnoitia* false-positive reactors, taking into account that *Sarcocystis* spp.

infection is present in almost 100% of cattle and that *N. caninum* infection is also highly prevalent depending on the region or country studied (Reichel et al., 2013). As the *B. besnoiti* in house ELISA employs a soluble tachyzoite extract, similar results could be obtained with other ELISAs based on similar antigenic extracts that already showed false positive results (Nasir et al., 2012). Thus, based on the results obtained herein, a higher number of sera from cattle infected with *Sarcocystis* spp. and or *N. caninum* should be included in the validation of serological assays to diagnose *B. besnoiti* infection. However, further comparative validation studies are essential for updating the diagnostic characteristics of all the available ELISA assays. Finally, the impact that *B. besnoiti* seropositivity may have on the diagnosis of bovine neosporosis should be the subject of further research.

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Objetivo 3. Identificación de nuevas dianas diagnósticas en la infección por *B. besnoiti* y desarrollo de una nueva prueba serológica.

Una de las principales limitaciones del diagnóstico serológico de la besnoitiosis bovina, es el elevado número de resultados falsos-positivos que se han puesto de manifiesto en diversos trabajos, principalmente cuando se emplean las técnicas de IFI y ELISA. En el Objetivo 2, estos resultados falsos-positivos se asociaron no solo a la presencia sino también a un elevado nivel de anticuerpos frente a *N. caninum* y *Sarcocystis* spp. Por ello, el Objetivo 3 comprende la identificación de nuevas dianas diagnósticas más específicas de *B. besnoiti,* así como la obtención y el empleo de nuevos extractos antigénicos que mejoren las características diagnósticas de las actuales pruebas. Con el fin de identificar nuevas dianas, se han realizado los siguientes estudios: *i)* la identificación mediante técnicas proteómicas de antígenos inmunodominantes y específicos del taquizoíto de *B. besnoiti*; *ii)* el estudio de las diferencias en abundancia de las proteínas y de su inmunogenicidad entre las dos especies del género *Besnoitia* - *B. besnoiti* y *B. tarandi* - que producen signos clínicos similares en el ganado bovino y en rumiantes silvestres, respectivamente; *iii)* el desarrollo y caracterización de MABs dirigidos frente a antígenos de taquizoíto de *B. besnoiti* y, finalmente *iv)* la obtención de un extracto antigénico basado en taquizoítos liofilizados de *B. besnoiti* y la evaluación de las características diagnósticas de un ELISA basado en este extracto para el diagnóstico de la besnoitiosis en bovinos y en rumiantes silvestres.

En el primer estudio, se ha descrito el inmunoma y el proteoma del estadio de taquizoíto de *B. besnoiti* mediante 2-DE inmunoblot y 2-DE, respectivamente, y se han identificado posibles dianas diagnósticas mediante 2-DE acoplada a MALDI-TOF/MS, respectivamente (Sub-objetivo 3.1). Entre los resultados obtenidos, cabe destacar que, en la descripción del proteoma, la mayoría de las manchas proteicas se localizaron en la zona ácida de un gradiente de pH 3-10 y entre 37-50 kDa, al igual que lo descrito en otros protozoos apicomplejos cercanos como *N. caninum* y *T. gondii*. El inmunoma mostró un

patrón similar al observado en el proteoma. Así, la mayoría de manchas antigénicas también se localizaron entre 37 y 50 kDa y en la zona ácida de un gradiente de pH 3-10. Las proteínas más abundantes en el proteoma de *B. besnoiti* fueron seleccionadas para su identificación. Un total de 27 manchas fueron correctamente identificadas, y correspondieron a 20 proteínas diferentes, indicando la presencia de diferentes especies de proteínas. Diez de las 20 proteínas identificadas fueron inmunogénicas. Desafortunadamente, todas actúan en procesos conservados, no solo en los parásitos apicomplejos, sino en general en todos los organismos eucariotas. En particular, cabe destacar que la mayoría corresponden a proteínas del metabolismo (fructosa 1,6-bisfosfatasa aldolasa, ENO, LDH y fosfoglicerato quinasa) y HSP60, HSP70 y HSP90. Por último, se han estudiado las reacciones cruzadas con el protozoo *N. caninum*, ya que se trata de un parásito muy cercano y es el agente etiológico de la neosporosis bovina, una enfermedad mundialmente distribuida, y cuya prevalencia en España es muy alta. Un pool de sueros procedente de vacas infectadas con *N. caninum* reconoció 25 manchas proteicas en el proteoma de *B. besnoiti,* de las que cinco fueron identificadas como fructosa 1,6-bisfosfatasa aldolasa, ENO, HSP60, HSP90 y actina, quedando por tanto descartadas como posibles dianas diagnósticas.

El estudio de las diferencias en abundancia de proteínas entre las dos especies de *Besnoitia* se ha realizado mediante la técnica proteómica 2-DE DIGE acoplado a MALDI-TOF/MS, mientras que las diferencias en su inmunogenicidad de las proteínas se han estudiado mediante 2-DE inmunoblot (Sub-objetivo 3.2). Cabe destacar que, si bien se han observado diferencias en abundancia en diversas manchas proteicas entre las dos especies estudiadas, el inmunoma reveló un patrón prácticamente idéntico entre *B. besnoiti* y *B. tarandi.* Las proteínas diferencialmente abundantes entre ambos proteomas fueron seleccionadas para su identificación mediante MALDI-TOF/MS. Desafortunadamente, y al igual que los resultados obtenidos en estudio anterior, todas las proteínas identificadas correspondieron a proteínas implicadas en procesos conservados en apicomplejos. Sin embargo, este estudio nos ha permitido identificar por primera vez proteínas como la GA3PDH y la purina nucleósido fosforilasa. Los resultados obtenidos

en estos dos estudios han evidenciado la dificultad para identificar antígenos específicos de especie con utilidad diagnóstica.

En paralelo, se han llevado a cabo otros abordajes para la obtención de dianas diagnósticas. En particular, se ha desarrollado un panel de ocho anticuerpos monoclonales (MABs) dirigidos frente a un extracto total y un extracto enriquecido en proteínas de membrana del taquizoíto de *B. besnoiti* (Sub-objetivo 3.3), que podrían resultar útiles tanto para el diagnóstico de la infección como para el estudio de la biología del parásito. Para co-localizar los epítopos reconocidos por los anticuerpos, se ha utilizado TEM así como la microscopía confocal. Además, se ha evaluado la especificidad de género, de especie y de estadio de los MABs mediante Western blot. Para ello, se han estudiado las reacciones cruzadas con taquizoítos de protozoos cercanos como *B. tarandi, N. caninum, T. gondii* y con cistozoítos de *Sarcocystis* spp., así como con el estadio de bradizoíto de *B. besnoiti.* Los resultados de este trabajo han demostrado que los MABs denominados 3.10.8 y 5.5.11 reconocieron la superficie de los taquizoítos de *B. besnoiti,* los MABs denominados 1.17.8, 8.9.2 y 2.G.A reconocieron el extremo apical de los taquizoítos y los MABs denominados 2.A.12, 2.F.3 y 2.G.4 reconocieron un contenido granular en el interior de los taquizoítos compatible con los gránulos densos. Cabe destacar que la mayoría de los MABs desarrollados fueron específicos de género. De hecho, ninguno reaccionó de forma cruzada con *T. gondii* y tan solo el MAB 2.F.3 reaccionó de forma cruzada con *Sarcocystis* spp. Desafortunadamente, las reacciones cruzadas con *N. caninum* tan solo pudieron ser evaluadas para los MABs 2.G.A, 2.A.12, 2.F.3 y 2.G.4 y todos mostraron resultados negativos. Por otra parte, todos los MABs excepto los MABs 1.17.8 y 2.G.A mostraron reacciones cruzadas con el estadio de taquizoíto de *B. tarandi.* Estos resultados concuerdan con los resultados observados en el Sub-objetivo 3.2 de la presente Tesis, en el que *B. besnoiti* y *B. tarandi* mostraron un patrón de reconocimiento de antígenos muy similar. Por último, todos los MABs desarrollados fueron específicos del estadio de taquizoíto de *B. besnoiti.* En base a estos resultados, los MABs específicos del taquizoíto de *Besnoitia* spp. 2.G.A, 2.A.12 y 2.G.4, junto con aquellos que reconocieron el extremo apical (1.17.8 y 8.9.2) y la superficie de los taquizoítos (3.10.8 y 5.5.11)

podrían ser buenos candidatos diagnósticos. Sin embargo, es esencial descartar la existencia de reacciones cruzadas entre los MABs 3.10.8, 5.5.11, 1.17.8 y 8.9.2 y *N. caninum.*

Por último, se ha obtenido un nuevo extracto antigénico basado en taquizoítos liofilizados de *B. besnoiti,* y se han evaluado las características diagnósticas de una prueba ELISA basada en este extracto (BbSALUVET ELISA 2.0) para el diagnóstico de la besnoitiosis en bovinos y en rumiantes silvestres (Sub-objetivo 3.4). Además, también se ha evaluado una prueba ELISA comercial (PrioCHECK Besnoitia Ab 2.0) y una prueba ELISA *in house* recientemente desarrollada (APure-BbELISA). Para ello, y en base a los resultados obtenidos en el Objetivo 2 de esta Tesis, se ha empleado un panel de sueros en el que las categorías de falsos-positivos y falsos-negativos estaban sobre-representadas. Los resultados han señalado que las tres pruebas evaluadas presentaron muy buenas características diagnósticas, si bien cabe destacar que APure-BbELISA y BbSALUVET ELISA 2.0 mostraron los mejores valores de Se y Esp. Además, la nueva prueba desarrollada fue validada con sueros de rumiantes silvestres, mostrando también muy buenas características diagnósticas. Por lo tanto, también puede resultar útil para realizar estudios epidemiológicos. En base a las buenas características observadas, así como por la fácil y escalable producción del extracto antigénico, se ha procedido a la protección del ELISA mediante una patente.

Sub-objective 3.1

First 2-DE approach towards characterising the proteome and immunome of *Besnoitia besnoiti* in the tachyzoite stage

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Abstract

Bovine besnoitiosis is caused by the cyst-forming apicomplexan parasite *Besnoitia besnoiti*. It is considered to be a re-emergent disease in Europe and is also present in Africa and Asia. Due to the chronic and debilitating course of the disease, bovine besnoitiosis is responsible for severe economic losses. However, many aspects of the disease and parasite biology remain unknown. Proteomics studies could help to investigate relevant biological processes as well as host immune response associated with parasite infection. Both the proteome and immunome of the tachyzoite stage of *B. besnoiti* of the Bb-Spain1 isolate are described herein for the first time. Tachyzoite protein extracts were first separated by 2-DE SDS-PAGE using pH 3-10 NL IPG strips for Coomassie Brilliant Blue-stained gels and immunoblots. Eighty-five out of 265 spots visualised on Coomassie-stained gels were immunogenic when pooled serum from naturally infected cattle was used, and the distribution of immunogenic spots correlated with the 1-DE IDA pattern. Because most spots were found in the acidic range of the pH gradient, pH 3-6 L IPG strips were used next, and 58 out of 123 visualised spots proved to be immunogenic. Twenty-seven spots were identified by MALDI TOF/TOF to be 20 different proteins due to the presence of protein species. All proteins identified corresponded to highly conserved proteins among eukaryotes. Six proteins identified are related to energy metabolism, 3 are heat shock proteins, 4 proteins are related to host cell invasion processes, and 2 proteins are involved in cell redox homeostasis. A tryptophanyl tRNA synthetase, a putative gbp1p, nucleoredoxin, a putative receptor for activated C kinase, and a nuclear movement domain-containing protein were also identified. Among these proteins, fructose-1,6-bisphosphate aldolase, lactate dehydrogenase, pyruvate kinase, enolase, HSP60, HSP70, HSP90, actin and profilin proved to be immunogenic, and 5 were cross-reactive antigens between *B. besnoiti* and *N. caninum*. This first proteomic approach carried out in *B. besnoiti* should be followed by other studies to identify more specific parasite proteins.

Keywords: *Besnoitia besnoiti;* Tachyzoite; Proteome; Immunome; 2-DE; Mass spectrometry.

1. Introduction

Bovine besnoitiosis is caused by the cyst-forming apicomplexan parasite *Besnoitia besnoiti* (Marotel, 1912), which belongs to the Toxoplasmatinae subfamily. This disease was first described in Southern France by Besnoit and Robin (1912) and was reported a few decades later in Sub-Saharan Africa and Asia (McCully et al., 1966; Neuman, 1972). An increase in the number of cases in the last 20 years has led to a geographic expansion of the disease in Western and Central Europe (Cortes et al., 2006c; Fernández-García et al., 2009b; Rostaher et al., 2010; Liénard et al., 2011; Manuali et al., 2011; Gentile et al., 2012). Bovine besnoitiosis is currently considered an emerging disease in Europe (EFSA, 2010; reviewed by Jacquiet et al., 2010; Mutinelli et al., 2011). Because there are no effective treatments or vaccines available, current control measures should focus on the detection of infected animals by both clinical inspection and serological analysis (García-Lunar et al., 2013a).

Both tachyzoite and bradyzoite parasite stages develop in the intermediate hosts (cattle and wild bovids; Pols, 1960; Basson et al., 1965). Tachyzoites are responsible for the acute phase of the disease because they invade the endothelia of blood vessels, causing increased vascular permeability and subsequent oedema, among other clinical signs. Chronic infection occurs in the pathognomonic phase of bovine besnoitiosis and is characterised by the development of subcutaneous tissue cysts containing bradyzoites. The cysts appear predominantly in subcutaneous tissue in the skin, mucosal membranes and sclera conjunctiva, and typical symptoms are cutaneous lesions such as thickening and folding of the skin and alopecia (Bigalke, 1981). Many aspects of the disease (e.g., prevalence, incidence and transmission routes) and parasite biology (e.g., definitive host, phylogeny and parasite-host cell interaction) remain to be elucidated (Diesing et al., 1988; reviewed by Olias et al., 2011).

Proteomics is a suitable platform to gain a better understanding of *B. besnoiti*. Proteomic tools have allowed for the investigation of relevant biological processes as well as host immune response associated with infections by other apicomplexan parasites of medical and veterinary importance such as *Eimeria tenella, Plasmodium falciparum, Neospora caninum* and *Toxoplasma gondii* (Lee et al., 2003; De Venevelles et al., 2004; Belli et al., 2005; Gelhaus et al., 2005; reviewed by Weiss et al., 2009). Proteomics studies on *B. besnoiti* could help to identify specific parasite proteins and/or antigenic proteins that could be considered as potential vaccine and/or diagnostic targets. *A priori* this task does not seem easy or straightforward because a whole sequenced genome for *B. besnoiti* is lacking. However, the availability of complete genomes of other members of the Toxoplasmatinae subfamily, such as *T. gondii* and *N. caninum,* in addition to the remarkable conservation of their genomes and gene expression (Reid et al., 2012), allow for the use of these genomes for the preliminary analysis of the global protein expression of *B. besnoiti*. The aim of this study was to conduct a proteomics study to describe the proteome and immunome of the tachyzoite stage of *B. besnoiti* using two-dimensional gel electrophoresis (2DE SDS-PAGE) and 2-DE immunoblotting coupled

with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and bioinformatics.

2. Materials and methods

2.1. Cell culture and tachyzoite purification

B. besnoiti tachyzoites from the BbSp-1 isolate (Fernández-García et al., 2009b) were grown in a Marc-145 cell monolayer with DMEM supplemented with 5% foetal calf serum. They were recovered from 3.5-day growth cultures when the majority of the tachyzoites were still intracellular and under exponential growth (at least 80% of undisrupted parasite vacuoles in the cell monolayer) to avoid potential variations in protein expression associated with the phase of the lytic cycle during collection. They were then purified using PD-10 desalting columns (GEHealthcare, Buckinghamshire, UK) as previously described Regidor-Cerrillo et al. (2012). Tachyzoite viability was confirmed by counting in a Neubauer chamber. Next, the tachyzoites were pelleted by centrifugation at 1,350 ×*g* for 10 min and stored at −80 ◦C until use. Microscope observations of the purified tachyzoites were carefully carried out to detect and discard parasite batches with host cell contamination.

Tachyzoites of *N. caninum* (Nc-1 isolate) (Dubey et al., 1988) and *T. gondii* (TgME-49 strain) (Lunde and Jacobs, 1963) were grown and purified as previously described (Fernández-García et al., 2009b) for use in serological assays.

2.2. Sera sample selection

To determine the immunome of the *B. besnoiti* tachyzoite stage, a pool of bovine serum samples seropositive against *B. besnoiti* was employed in 2-DE immunoblots. This pool was composed of 7 sera obtained from cattle naturally infected with *B. besnoiti* and showing characteristic clinical signs (visible tissue cysts in conjunctiva and *vestibulum vaginae*). These animals came from a herd with a previous history of besnoitiosis. Positive sera were selected based on IFAT, ELISA and Western blot results (García-Lunar et al., 2013a). The pool was checked against *B. besnoiti* by tachyzoite-based immunoblotting and titrated with IFAT (1:800) (Fernández-García et al., 2009a). All samples were also checked against *N. caninum* and *T. gondii* extracts by tachyzoite-based Western blotting (Álvarez-García et al., 2002; Chávez-Velásquez et al., 2005) to avoid cross-reactions and proved to be negative.

As a negative control, a pool of 5 bovine serum samples without clinical signs and seronegative to *B. besnoiti* by IFAT, ELISA and tachyzoite-based Western blotting were selected. The pool of negative sera was checked against *B. besnoiti*, *N. caninum* and *T. gondii* infection by Western blotting and proved to be negative.

To evaluate possible cross-reactions, a pool of 5 serum samples collected from cattle naturally infected by *N. caninum* was also used. The samples were obtained from a herd free of *Besnoitia* spp. infection and with high intra-herd seroprevalence of bovine neosporosis (approximately 85%) and a 5% abortion rate (Rojo-Montejo et al., 2009). This pool of sera was shown to be seronegative for *B. besnoiti* by IFAT and Western blotting and was titrated with *N. caninum* IFAT (1:800) (Álvarez-García et al., 2002).

2.3. Protein extracts from B. besnoiti tachyzoites

Protein extraction was performed as described by Regidor-Cerrillo et al. (2012) with limited modifications. Approximately 3×10^8 frozen tachyzoites were disrupted in 150 µl of lysis buffer containing 6 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1propanesulfonate (CHAPS), 65 mM 1,4-dithioerythreitol (DTE), 10 mM Tris-HCl pH 7, and 1 mM phenylmethanesulphonyl fluoride (PMSF) (added fresh) and subjected to 3 cycles of rapid freezing and thawing using liquid nitrogen. Tachyzoites were further disrupted by sonication for 15 min at 4 ◦C in a water bath sonicator (Ultrasonics Ltd.). Solubilisation was aided by the subsequent addition of 150 µl of rehydration buffer containing 8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 65 mM DTE, and 1% ampholyte. Insoluble material was removed by centrifugation at 13,000 ×*g* for 30 min at 4 ◦C. Protein concentrations of the resulting supernatants were determined by the Bradford method using bovine serum albumin as a standard.

2.4. 2-DE SDS-PAGE

For 2-DE SDS-PAGE with Coomassie brilliant blue (CBB)-stained gels (Kang et al., 2002), 400 µg of protein was applied diluted in 300 µl of lysis and rehydration buffer (1:1) to Immobiline pH 3-10 NL and pH 3-6 L IPG strips (17 cm) (Bio-Rad). For immunoblotting, only 100 µg of protein was loaded in each strip. Isoelectric focusing (IEF) was performed at 20 ◦C with a Protean IEF cell system (Bio-Rad) by passive rehydration for 16 h. The first dimension was carried out using a program of 500 V for 2 h, 500-2000 V for 4 h, 200-4500 V for 6 h and 4500 V for 17 h. Three replicates were carried out for both the staining and immunoblotting of each pH strip. Strips were either processed immediately for second-dimension electrophoresis or stored at −80 ◦C until use.

Before the second-dimension separation, proteins on the strips were reduced with 4% DTE and then alkylated with 5% iodoacetamide in equilibration buffer (6 M urea, 50 mM Tris-HCl pH 6.5, 30% glycerol, and 2% SDS). Second-dimension electrophoresis was performed using 10% polyacrylamide native gels containing 2.5 M Tris pH 8.9, 0.16 M PDA, 0.1% SDS, 0.1% PSA, and 3.8 mg/ml sodium thiosulfate using electrophoresis buffer (0.025 M Tris, 0.192 M glycine, and 0.1% SDS) at 45-50 mA per gel for 6 h at 18 ◦C with a Protean II xi Cell (Bio-Rad). A marker (Precision Plus Kaleidoscope, Bio-Rad Laboratories) ranging from 250 to 10 kDa was used to follow the separation and to estimate molecular weights. After separation, proteins in the 400 µg gels were visualised by CBB G250 (Bio-Rad) (Kang et al., 2002) for the proteomics analysis, and the 100 µg gels were transferred to PVDF membranes (Immobilon TM-P, Millipore, Bedford, USA) for immunoblotting, as described in Section 2.5.

2.5. Immunoblotting

After 2-DE SDS-PAGE separation, 3-10 NL and 3-6 L IPG strips were transferred at 18 ◦C onto PVDF membranes (Immobilon TM-P, Millipore, Bedford, USA) for immunoblotting using transfer buffer (0.25 M Tris, 1.92 M glycine, and 1% methanol). The blotted membranes were blocked with TBS-T buffer [10 mM Tris-HCl, 150 mM NaCl, and 0.05% (v/v)

Tween-20 pH 7.2] containing 5% (w/v) horse serum for 1.5 h at room temperature (RT). The PVDF membranes were washed for 1 h with TBS-T and then incubated with primary antibody (anti-*B. besnoiti* bovine pool sera) diluted at 1:800 according to their titre, as established by IFAT for 1.5 h at RT. After being washed for 1 h with TBS-T, the membranes were incubated with the secondary antibody, an anti-bovine IgG1/IgG2 antibody conjugated with peroxidase (9D8-P Laboratories LSI, Lissieu, France), at a 1:10,000 dilution for 1.5 h at RT. Immunoblots were exposed for 1-5 min using the Immobilon Western Chemiluminescent HRP Substrate, a chemiluminescence method (Millipore, Billerica, MA, USA). For image acquisition, AGFA CP1000 processor and AGFA films (Curix/RP2 Plus, 18 cm × 24 cm) were used.

For the cross-reaction study, 2-DE SDS-PAGE immunoblotting was performed with PVDF membranes and pH 3-10 NL IPG strips (17 cm) (Bio-Rad) using pooled serum positive to *N. caninum* infection at a 1:800 dilution according to the titre established by IFAT.

2.6. Image capture and matching

All spots detected by the PDQuestTM (Bio-Rad) program were manually verified, and false-positive and false-negative spots were manually removed or added to the images, respectively. To compare spots, a MatchSet was created from the images of the gels and immunoblots. A standard gel (master) was created with the spot data from all of the image replicates for CBB and immunoblotting. The automated matching tool of the PDQuestTM software (Bio-Rad) was used to match spots across gels. When a spot was present/absent in at least two replicate gels or immunoblots was considered to be reproducibly present/absent. All spots matched by the software program were manually verified. All gels and immunoblots performed in the study were highly reproducible, as matches rates varied from 80 to 100%.

2.7. Mass spectrometry analysis (MS MS/MS)

Abundant spots were excised from CBB-stained gels, and MS analysis was carried out as described Regidor-Cerrillo et al. (2012). After digestion, the supernatant was collected, and 1 µl was spotted onto a stainless steel MALDI plate and allowed to air-dry at RT. Then, 0.4 µl of a 3 mg/ml of a-cyano-4-hydroxycinnamic acid (CHCA; Sigma-Aldrich, St. Louis, MO, USA) in 50% acetonitrile (CAN) and 0.1% trifluoroacetic acid (TFA) was added to the dried peptide digest spots and again allowed to air-dry at RT. MALDI-TOF MS analysis was performed with a matrix-assisted laser desorption/ionisation (MALDI) time-of-flight (TOF) tandem mass spectrometer (4800 plus MALDI-TOF/TOF Analyzer; Applied Biosystems; MDS Sciex, Toronto, Canada) at the Proteomic Unit, Complutense University of Madrid operated in reflector mode with an accelerating voltage of 20,000 V. All mass spectra were calibrated internally using peptides from the autodigestion of trypsin (sequencing grade; Roche Molecular Biochemicals). For mass fingerprint (PMF) identification, monoisotopic peptide masses observed with a signal to noise greater than 10 were compared with the NCBI nr (24.02.2011: 13135398 sequences; 4494708239 residues), and ToxoDB release 7.1 (*N. caninum* (Nc-Liverpool isolate):

7082 sequences; 6037978 residues, *T. gondii*: TgME-49: 7993 sequences; 5950736 residues and TgVEG: 7846 sequences; 5484338 residues) (Gajria et al., 2008) databases using the MASCOT algorithm v2.3 (http://www.matrixscience.com/). The peptide fingerprint for each identified spot is shown in Supplementary Data SD1 according to MIAPE guidelines.

For MS/MS sequencing analyses, suitable precursors were selected and fragmented using CID (with atmospheric gas) with 1 Kv ion reflector mode and precursor mass windows of ±5 Da. The plate model and default calibration were optimised for MS/MS spectra processing.

For protein identification, MASCOT was used to search through the Global Protein Server v. 3.6 from Applied Biosystems with the following parameters: carbamidomethyl cysteine as a fixed modification; oxidised methionine as a variable modification; peptide mass tolerance of 50 ppm for PMF and 80,100 ppm for the MS/MS search; 1 missed trypsin cleavage site; and MS/MS fragment tolerance of ±0.3 Da. The parameters for the combined search (peptide mass fingerprint plus MS/MS spectra) were the same as described above. Identification was accepted when the probability score was greater than the score fixed by MASCOT as significant with a p value less than 0.05. Correlation of the gel region with a predicted molecular weight and p*I* was also considered for protein identification. Protein information such as biological process, molecular function (identified with GO terms) and pathways was obtained from the ToxoDB 7.1 database (Gajria et al., 2008).

3. Results and discussion

Proteomics approaches have been widely employed with several apicomplexan parasites (*N. caninum*, *E. tenella*, *P. falciparum* and *T. gondii*) (Lee et al., 2003; De Venevelles et al., 2004; Gelhaus et al., 2005; Ma et al., 2009). Initial studies provided descriptions of their proteomes as well as their immunomes. The availability of the entirely or partially sequenced genomes of cyst-forming coccidia parasites of medical and/or veterinary importance (*T. gondii* and *N. caninum*) greatly facilitated further studies on differences in protein expression and in immunogenic profiles among different species (Lee et al., 2005; Zhang et al., 2011), isolates (Shin et al., 2005; Regidor-Cerrillo et al., 2012) or parasite stages (Marugán-Hernández et al., 2010). These methods have allowed the study of the mechanisms of host cell invasion, the composition of specialised secretory organelles, stage conversion and organisation of the cytoskeleton (reviewed by Weiss et al., 2009).

3.1. Proteome of the tachyzoite stage of B. besnoiti

Approximately 265 ± 18 spots of *B. besnoiti* tachyzoites were observed on the CBB-stained gels using pH 3-10 NL IPG strips (17 cm) (Bio-Rad) (Fig. 1A). Although the spots detected in this study were located at p*I* values between pH 5.07 and pH 9.15 and had Mr values ranging from 13.168 to 77.525 kDa (Table 1), most of the abundant spots were found between 37 and 50 kDa and in the acidic range of the pH gradient. A similar spot distribution was observed for *N. caninum* and *T. gondii* (Cohen et al., 2002; Lee et al., 2003). To increase the resolution of the acidic region,

protein extracts were resolved using narrow range gels by using pH 3-6 L IPG strips (17 cm) (Bio-Rad). Using this technique, 123 ± 31 spots of *B. besnoiti* tachyzoites were observed on the CBB-stained gels; most of the spots had Mr values ranging from 17.829 to 97.281 kDa and p*I* values ranging from pH 4.40 and 6.06 (Fig. 2A). Although previous studies in other Sarcocystidae (Cohen et al., 2002; Lee et al., 2003) are not comparable because different conditions, such as the amount of protein loaded per gel, the strip pH range, and the staining procedure, were used, a larger number of proteins between 37 and 50 kDa and at the acidic range of the pH gradient for both of the *T. gondii* and *N. caninum* parasites were detected.

Fig. 1. Proteome (A) and immunome (B) profile of the tachyzoite stage of *B. besnoiti*.

Proteins were separated along a non-linear pH gradient (pH 3-10 NL IPG strips; 17 cm) in the first dimension and on a 10% polyacrylamide gel in the second dimension. Gels were stained with Coomassie Brilliant Blue for the proteome or transferred to PVDF membranes for the immunome. 2-DE gel and immunoblot images were analysed with PDQUEST software. Proteins that were identified by MS analysis are marked with arrows. Excised proteins for MS analysis that were not identified are marked with a circle.

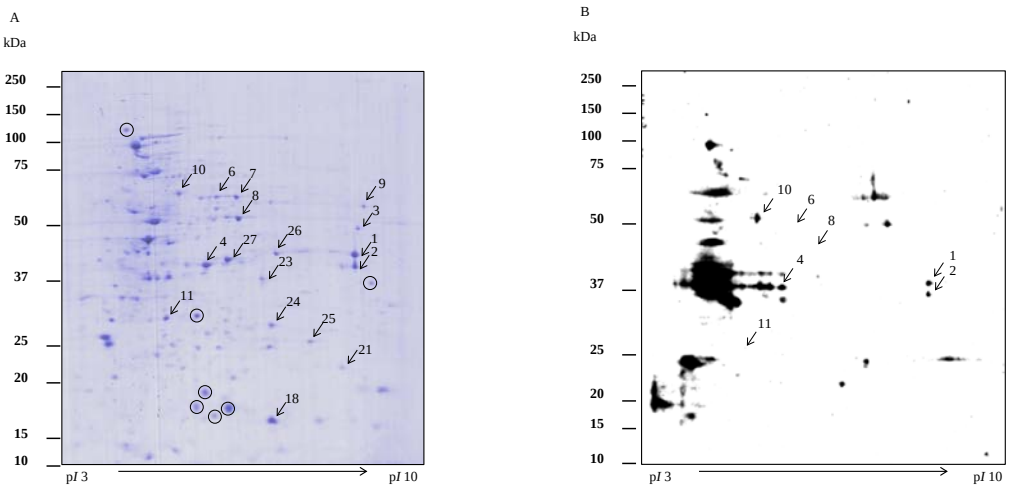
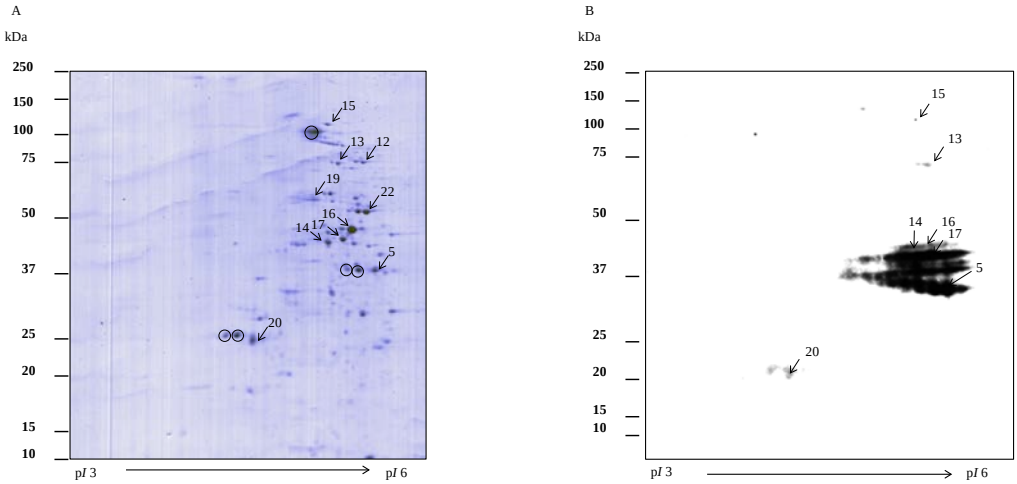


Fig. 2. Proteome (A) and immunome (B) profile of the tachyzoite stage of *B. besnoiti*.

Proteins were separated along a linear pH gradient (pH 3-6 L IPG strips; 17 cm) in the first dimension and on a 10% polyacrylamide gel in the second dimension. Gels were stained with Coomassie Brilliant Blue for the proteome or transferred to PVDF membranes for the immunome. 2-DE gel and immunoblot images were analysed with PDQUEST software. Proteins that were identified by MS analysis are marked with arrows. Excised proteins for MS analysis that were not identified are marked with a circle.



3.2. Immunome of the tachyzoite stage of B. besnoiti

For describing the immunome of the tachyzoite stage of *B. besnoiti*, a pool of bovine sera was used to avoid possible individual variations in the immune response. This pool was obtained from cattle naturally infected with *B. besnoiti* to identify relevant antigens as diagnostic targets in natural infections. In previous work, several immunogenic regions were recognised by 1-DE immunoblotting (Fernández-García et al., 2009a; Schares et al., 2010), a technique recommended for re-testing valuable animals with ambiguous results (García-Lunar et al., 2013a). Approximately 85 spots were detected by immunoblotting using pH 3-10 NL IPG strips (17 cm) (Bio-Rad) (Fig. 1B). As for the proteome, most spots were located between 50 and 37 kDa and in the acidic range of the pH gradient. Narrow gels of pH 3-6 L IPG strips (17 cm) (Bio-Rad) were used to increase the resolution of this region, and 58 spots were visualised (Fig. 2B).

Most immune-proteomics studies carried out in apicomplexan parasites have employed anti-rabbit polyclonal sera (Ma et al., 2009) or sera obtained from experimentally infected cattle (Shin et al., 2005) and have provided important information about the *T. gondii* and *N. caninum* immunogenic profiles. However, sera obtained from naturally infected individuals are the best samples for determining diagnostic targets. Unfortunately, this strategy has been rarely used (Geissler et al., 1999). In the present study, 2-DE SDS-PAGE immunoblotting was performed using sera from naturally infected cattle, and immunogenic antigens were mainly located at Mr ranges between

75 and 50 kDa, 37 and 50 kDa and 20 and 25 kDa, similar to the results found for *N. caninum* and *T. gondii* (Shin et al., 2005; Ma et al., 2009). In addition, the distribution of immunogenic spots correlates with the 1-DE IDA pattern of the tachyzoite stage of *B. besnoiti* described by Fernández-García et al. (2009a), where six IDAs (14.2, 33, 37.1, 39.6, 46.3 and 190.8 kDa) were identified.

3.3. Protein identification

The most abundant spots (24 from the pH 3-10 NL strips and 15 from the pH 3-6 L strips) were excised from CBB-stained gels for protein identification by MS analysis (Figs. 1 and 2). Using this strategy, the spectral data were compared against the NCBI nr database and 12 spots were identified as *Besnoitia*, *Neospora* or *Toxoplasma* proteins. The number of identifications increased to 20 when the mass fingerprints were compared against the ToxoDB 7.1 database (Supplementary Table ST1, Supplementary Data SD1). MALDI-TOF/TOF data were used to supplement the PMF data and to assign identities to 7 new spots, as well as to confirm 6 proteins previously identified by mass fingerprinting (Supplementary Table ST1). One spot identified by PMF was not confirmed by MALDI-TOF/TOF and was not included in the study. Peptide identifications were generated successfully for 27 spots (identifications with the highest scores are shown in Table 1). These 27 spots correspond to 20 different proteins, indicating the presence of multiple protein species, which may include isoforms, post-translational modifications and elements of protein processing. Only one out of the 20 identified proteins corresponds to a specific *B. besnoiti* protein, *B. besnoiti* protein disulphide isomerase (BbPDI), which may be explained by the fact that *B. besnoiti* genome has not been sequenced yet and there are only a few *B. besnoiti* ESTs currently available. Recent studies carried out on the molecular phylogeny of coccidian parasites have indicated that the *Besnoitia* genus belongs to Toxoplasmatinae subfamily (Ellis et al., 2000). Thus, successful identification of specific proteins of *B. besnoiti* was made based on homology with sequences available in databases from other members of the Toxoplasmatinae subfamily such as *N. caninum* (Nc-Liverpool isolate) or *T. gondii* (TgVEG and TgME49 strains).

Six out of 20 proteins identified in this study are related to energy metabolism, fructose-1,6 bisphosphate aldolase, putative phosphoglycerate kinase, lactate dehydrogenase (LDH), pyruvate kinase, enolase 2 protein (ENO), and putative ATP synthase alpha chain. Moreover, 3 heat shock proteins (HSPs; HSP60, HSP70 and HSP90), 4 proteins involved in host cell invasion (actin, actin depolymerising factor, putative tubulin β chain and profilin family protein), 2 proteins involved in cell redox homeostasis (thioredoxin-dependent peroxide reductase and protein disulphide isomerase (PDI)) were identified together with putative tryptophanyl tRNA synthetase, gbp1p, nucleoredoxin putative receptor for activated C kinase and nuclear

Table 1. Summary of proteins in the tachyzoite stage of *B. besnoiti* identified by MS and/or MS/MS.

Spot nº	Identified protein	Protein score	The. MWS (kDA)	The. p*I*	Nº matched peptides or amino acid sequence of peptides	Sequence coverage (%) [c]	Accession nº	Functional category
1	Fructose-1,6-bisphosphate aldolase [d, e]	150	47.387	9.01	19/65 [a]	39%	gb|TGME49_036040	Glycolytic enzime [f]
2	Fructose-1,6-bisphosphate aldolase [*Toxoplasma gondii*] [d]	104	39.357	7.63	K.GKPTNLSITEVAHGLAR.Y (63) [b]	16%	gi|25989716	Glycolytic enzime [h]
3	Phosphoglycerate kinase, putative	80	45.103	6.57	R.CLVLLSHAGRPDGR.V (31) [b] R.CLVLLSHAGRPDGR.V (39) [b]	24%	gb|TGME49_118230	Glycolytic enzime [f]
4	Lactate dehydrogenase (LDH) [d]	257	36.095	6.03	R.YVADALSVSPR.D (118) [b]	50%	gb|TGME49_032350	Glycolytic enzime [f]
5	Chain A, *T. Gondii* Bradyzoite-Specific LDH (LDH1) In Complex With Nad And Oxq [d]	109	36.047	6.06	14/65 [a]	39%	gi|310689950	Glycolytic enzime [h]
6	Chain A, Crystal Structure Of Pyruvate Kinase From *T. gondii*, 55.M00007 [d]	147	56.455	6.00	23/65 [a]	38%	gi|209447575	Glycolytic enzime [h]
7	Pyruvate kinase, putative	148	58.120	6.01	24/65 [a]	38%	gb|TGME49_056760	Glycolytic enzime [f]
8	Enolase 2 (ENO) [d, e]	93	52.765	6.43	14/65 [a]	32%	gb|TGME49_068850	Glycolytic enzime [f]
9	ATP synthase alpha chain, putative	170	61.472	8.84	R.ELIIGDRQTGK.T (36) [b] R.EAYPGDVFYLHSR.L (70) [b]	24%	gb|TGME49_004400	ATP synthesis coupled proton transport [f]
10	Heat shock protein 60 (HSP60) [d, e]	233	61.388	5.71	K.QVASTTNDIAGDGTTTATLLAR.A (94) [b] K.AVDAGMNPMDLLR.G (71) [b] K.AVDAGMNPMDLLR.G (26) [b]	28%	gb|TGME49_047550	Cellular protein metabolic process [f] Chaperone, stress response [h]
11	Heat shock protein 70, putative (HSP70) [d]	139	73.291	5.07	21/65 [a]	23%	gb|TGME49_073760	Chaperone, stress response [h]
12	Heat shock protein 70, putative [*T. gondii* ME49] (HSP70)	147	73.379	5.23	22/65 [a]	35%	gi|237830213	Chaperone, stress response [h]
13	Heat shock protein 70, putative (HSP70) [d]	157	73.070	5.07	22/65 [a]	39%	psu|NCLIV_033950	Chaperone, stress response [h]
14	Heat shock protein 70 [*T. gondii*] (HSP70) [d]	96	68.886	5.17	16/65 [a]	28%	gi|5738968	Chaperone, stress response [h]
15	Heat shock protein 90, putative (HSP90) [d, e]	142	97.281	4.91	R.FSQFMSYPIYVR.T (60) [b]	25%	psu|NCLIV_019110	Chaperone, stress response [f]

16	Actin [d, e]	250	42.166	5.05	28/65 [a]	62%	gb|TGME49_009030	Protein binding [g] Invasion and gliding motility [h]
17	Actin [d, e]	223	42.166	5.05	26/65 [a]	61%	gb|TGME49_009030	Protein binding [g] Invasion and gliding motility [h]
18	Actin depolymerising factor, putative	197	13.168	7.63	R.FGVYDCGNK.I (70) [b] K.IQFVLWCPDNAPVKPR.M (107) [b]	39%	gb|TGVEG_087130	Invasion and gliding motility [h]
19	Tubulin beta chain [*T. gondii* ME49]	215	50.554	4.70	28/65 [a]	53%	gi|237838955	Microtubule-based process [h]
20	Profilin family protein [d]	164	17.829	4.40	R.TSALAFAEYLHQSGY.- (137) [b]	30%	gb|TGME49_093690	Cytoskeleton organization [f]
21	Thioredoxin-dependent peroxide reductase, mitochondrial, related	157	22.174	5.96	R.LQGEFEKR.G (60) [b] K.ISFPLLADVSHK.M (88) [b]	10%	psu|NCLIV_062630	Cell redox homeostasis [f]
22	Protein disulphide isomerase, putative	202	53.299	5.22	23/65 [a]	51%	gi|94962169	Cell redox homeostasis [h]
23	Tryptophanyl-tRNA synthetase, putative [*T. gondii* GT1]	110	77.525	6.91	18/65 [a]	21%	gi|221484460	Translation [h]
24	Gbp1p protein, putative	152	31.911	9.15	R.SKGCGIVEYTNVEDAQK.A (126) [b]	19%	gb|TGME49_062620	Nucleic acid binding [g]
25	Nucleoredoxin, putative	137	25.008	5.99	K.SVALYFADGADPK.C (106) [b]	25%	gb|TGVEG_023230	-
26	Receptor for activated C kinase, RACK protein, putative	331	35.752	5.89	R.SFQGHTSDVNSVAFSPDNR.Q (159) [b] K.NVLSEITPEKTNR.S (66) [b]	39%	gb|TGME49_016880	-
27	Nuclear movement domain-containing protein	32	30.191	7.72	K.MIPGGVGDMSLLK.K (29) [b] K.MIPGGVGDMSLLK.K (29) [b]	4%	gb|TGVEG_070650	-

This table was made following the MIAPE guidelines developed by (HUPO-PSI) (Version 2.97.6, 10th February, 2012).

[a] Number of peptide mass values search/matched.

[b] Amino acid sequence identified by MS/MS; the ion score is indicated in parentheses.

[c] Amino acid sequence coverage for the proteins identified by MS and MS/MS.

[d] Immunogenic proteins.

[e] Cross-reactive antigens between *B. besnoiti* and *N. caninum*.

[f] Biological function (GO function).

[g] Molecular function (GO function).

[h] Function described in previous studies.

movement domain-containing protein (Table 1).

Immunoblotting and CBB-stained gels carried out with pH 3-10 NL strips (17 cm) (Bio-Rad) were compared by PDQuest analysis, and 7 out of the 24 spots excised from the CBB-stained gel were reactive against the bovine pooled serum, as indicated by 2-DE SDS-PAGE and immunoblotting. In the case of pH 3-6 L IPG strips (17 cm) (Bio-Rad), 7 out of the 15 spots excised from the CBB-stained gel were immunogenic (Figs. 1B and 2B). This method led to the identification of 14 spots, corresponding to 9 different proteins. The immunogenic proteins identified were 4 proteins related to energy metabolism (fructose-1,6-bisphosphate aldolase, LDH, pyruvate kinase and ENO), 3 heat shock proteins (HSP60, HSP70 and HSP90), actin and profilin (Table 1 and Figs. 1B and 2B).

All of the identified proteins correspond to highly conserved proteins involved in metabolic pathways and are commonly described in proteomics studies (Cohen et al., 2002; Lee et al., 2003; Ma et al., 2009; Marugán-Hernández et al., 2010). As expected based on sequence analogy with the closely related *T. gondii* and *N. caninum*, most of the proteins identified in this study are related to metabolism. Contrary to our expectations no proteins related to the tricarboxylic acid cycle were identified in this study, whereas 5 glycolytic enzymes were found. Information provided by the ToxoDB 7.1 database revealed the presence of 2 genes encoding 2 fructose 1,6 bisphosphate proteins in both *T. gondii* and *N. caninum*. In this study, 2 spots were identified corresponding to both *T. gondii* isoforms, which may suggest the existence of these two different isoforms for this protein in the tachyzoite stage of *B. besnoiti*.

Moreover, two differentially expressed stage isoforms of LDH and ENO have been reported in *T. gondii*: LDH 1 and ENO 2 appear in the bradyzoite stage and LDH 2 and ENO 1 appear in the tachyzoite stage (Yang and Parmey, 1997). Furthermore the bradyzoite ENO of *T. gondii* has been reported to be over expressed in the bradyzoite stage of *N. caninum* (Marugán-Hernández et al., 2010). In this study, one spot was identified as bradyzoite LDH 1 of *T. gondii*. This spot was also identified as malate dehydrogenase (MDH) of *N. caninum* (Supplementary Table ST1). This may be explained by the fact that MDH and a variety of LDHs share high sequence homology and belong to the NAD (P)-dependent dehydrogenase superfamily. In contrast, the identified ENO protein corresponds to the *T. gondii* ENO over-expressed in the tachyzoite stage.

Stress has been shown to induce bradyzoite formation, and HSPs are likely to play an important role during stage conversion. In this study, three antigenic HSPs commonly described in *T. gondii* and *N. caninum* in proteomic studies have been identified (HSP60, HSP70 and HSP90) (Weiss et al., 1998; Marugán-Hernández et al., 2010; Echeverria et al., 2010). Regarding HSP70, it is a highly abundant protein in apicomplexan parasites and is often detected in proteomics studies (Cohen et al., 2002; Shin et al., 2004; Marugán-Hernández et al., 2010) with different isoforms (Lee et al., 2003). HSP70 was identified in 4 different spots in this study, 2 of which were at similar MWS and p*I* values as its orthologues in *T. gondii* and *N. caninum* (MWS ranging from 73 to 73.3

and p*I* 5.0-5.2) but the other 2 were at different Mr values (Table 1 and Figs. 1A and 2A). Moreover only 3 out of these 4 spots proved to be immunogenic (Figs. 1B and 2B). According to the information provided by MS identification, the spots identified as HSP70 may be considered protein species potentially encoded by at least 2 genes.

B. besnoiti, similar to other apicomplexans, may rely on gliding motility for active invasion. Indeed 4 proteins related to this activity were identified: actin, actindepolymerising factor, profilin and tubulin β chain but only actin and profilin proved to be immunogenic. Actin was identified in 2 antigenic spots exhibiting similar MWS and p*I* values. The regulation of actin turnover is critical for maintaining proper filamentous networks within cells. Therefore, apicomplexan parasites have evolved numerous actin-binding proteins to ensure proper regulation of actin polymerisation. Concerning this fact, profilins are small monomeric actin-binding proteins that play multiple roles in regulating actin polymerisation and have recently been demonstrated to play an important role in gliding motility in *T. gondii* (Skillman et al., 2012).

Regarding proteins involved in cell redox homeostasis, two cytoplasmic protein members of the thioredoxin superfamily (Carvalho et al., 2006) were identified: PDI and thioredoxin-dependent peroxide reductase. Concerning PDI, it is a key enzyme that enables proteins to acquire their correct three-dimensional structure. Parasite PDI-like enzymes have been previously described in *N. caninum* (Naguleswaran et al., 2005) and *T. gondii* (Meek et al., 2002). Their orthologue in *B. besnoiti* is the only protein that has been identified and characterised to date (Marcelino et al., 2011) and phylogenetic analyses confirmed that *B. besnoiti* PDI (BbPDI) is closer phylogenetically to the PDIs from *N. caninum* and *T. gondii*.

Finally two proteins related to protein synthesis were identified: tryptophanyl-tRNA synthetase and Gbp1p, a protein that binds single-stranded telomeric DNA. Tryptophanyl-tRNA synthetase was previously identified in the proteome map of both *T. gondii* and *N. caninum* (Zhang et al., 2011).

3.4. Cross-reactions

It is widely known that there are cross-reactive antigens among members of the Sarcocystidae family (Cortes et al., 2006a; Fernández-García et al., 2009a; Schares et al., 2010) that may interfere with serological diagnoses. As bovine neosporosis is distributed worldwide and high seroprevalence rates have been reported in Europe (Bartels et al., 2006) where bovine besnoitiosis is also present, cross-reactions between *N. caninum* and *B. besnoiti* should be investigated. In fact, Shkap et al. (2002) described that anti-*N. caninum* sera reacted by IFAT with an *B. besnoiti* antigen at low IFAT titres. Because all of the proteins identified in this study are conserved, cross-reactions with *N. caninum* sera samples were further investigated. Twenty-five spots identified by immunoblotting with a pool of bovine sera from *N. caninum*-infected cattle were matched to *B. besnoiti* spots and 5 corresponded to previously identified proteins (fructose-1,6-bisphosphate aldolase, ENO, HSP60, HSP90, and actin). Although fructose 1,6 bisphosphate aldolase was identified in two different spots, only

one showed cross-reactions with anti-*N. caninum* sera (Table 1). Cross-reactions between the closely related *T. gondii* and *N. caninum* have been previously reported. Lee et al. (2005) employed polyclonal sera produced in rabbits to describe the *N. caninum* immunome, and HSP70, tubulin β chain, PDI, actin and ENO were believed to be conserved antigens in both *T. gondii* and *N. caninum*. Moreover, despite the fact that Zhang et al. (2011) employed polyclonal anti-*T. gondii* and anti-*N. caninum* sera produced in mice, dense granule and microneme proteins, heat shock proteins, glycolytic enzymes, and cytoskeletal proteins were found to be species-specific proteins, whereas fructose 1,6 bisphosphate aldolase, ENO and PDI among others were identified as cross-reactive antigens. Strong cross-reactions between *B. besnoiti* and *B. tarandi* (Gutiérrez-Expósito et al., 2012) and between *B. besnoiti* and *B. bennetti* (Ness et al., 2012) have also been reported using different serological tools.

In conclusion, this is the first proteomics approach to describe both the proteome and immunome of the tachyzoite stage of *B. besnoiti*. In this work, 20 proteins (9 immunogenic) were successfully identified, even in the absence of the genome sequence of *B. besnoiti* and 5 of them showed cross-reactions with *N. caninum*. A low number of diagnostic targets were identified in this study because most antigens were conserved proteins among apicomplexan parasites. Only well-visualised proteins in CBB stained gels were excised for MS identification in order to avoid identification failure due to a low amount of protein. Thus, the identification of the immunogenic spots may be biased towards the most abundant ones in this study. In addition, surface antigens and proteins from secretory organelles, which have been previously identified in other members of family Sarcocystidae, were not identified in the present study. As these proteins are highly immunogenic, they are considered good candidates for diagnosis (Saadatnia et al., 2012). However, due to the hydrophobicity of membrane proteins and, probably, less abundance of secreted proteins, additional approaches are needed to overcome these obstacles and to identify valuable targets (Bradley et al., 2005; reviewed by Bradley and Sibley, 2007; Marugán-Hernández et al., 2010; Che et al., 2011; Luo et al., 2011). Moreover, considering the lack of the genome sequence of *B. besnoiti* together with the fact that genes encoding for proteins related to invasion may have diverged as for *N. caninum* and *T. gondii* (Reid et al., 2012), these may have led to the failure in the identification of these proteins. The present study sets a starting point to answer specific questions suggested by others such as the relationship between different *Besnoitia* species that affects ungulates (reviewed by Olias et al., 2011; Gutiérrez-Expósito et al., 2012) as well as differences between parasite stages of *B. besnoiti*. Related to this, conversion from tachyzoite to bradyzoite needs to be further investigated since the development of serological assays based on antigenic stage-specific proteins may improve the serological diagnosis of bovine besnoitiosis. However, further research should give priority to sequencing of the whole *B. besnoiti* genome, as data generated by means of proteomic tools would be more fruitful and could be integrated into gene annotation systems (Xia et al., 2008).

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2012.12.040.

Sub-objective 3.2

Proteomics reveals differences in protein abundance and highly similar antigenic profiles between *Besnoitia besnoiti* and *Besnoitia tarandi*

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Abstract

Besnoitia besnoiti and *Besnoitia tarandi* are two cyst-forming apicomplexan parasites of the genus *Besnoitia*. *B. besnoiti* uses cattle as an intermediate host, in which it causes a disease that progresses in two sequential phases: the acute anasarca stage and the chronic scleroderma stage. Reindeer and caribou act as intermediate hosts for *B. tarandi*, which causes clinical signs similar to those caused by *B. besnoiti*. Previous studies demonstrated high molecular similarity, as determined by 18S and ITS-1 RNA sequences, between these *Besnoitia* spp., and strong serological cross-reactivity between these species has recently been demonstrated. Thus, a difference gel electrophoresis approach and mass spectrometry analysis were used to describe the proteomes and explore differences in protein abundance between *B. besnoiti* and *B. tarandi* in tachyzoite extracts. Immunoproteomes were also compared using 2-DE immunoblotting with polyclonal sera from experimentally infected rabbits. From approximately 1,400 spots detected in DIGE-gels, 28 and 29 spots were differentially abundant in *B. besnoiti* and *B. tarandi* tachyzoites, respectively (±1.5-fold, $p < 0.05$). Four and 13 spots were exclusively detected in *B. besnoiti* and *B. tarandi*, respectively. Of the 32 differentially abundant spots analyzed by MALDI-TOF/MS, 6 up-regulated *B. besnoiti* proteins (LDH; HSP90; purine nucleoside phosphorylase and 3 hypothetical proteins) and 6 up-regulated *B. tarandi* proteins (G3PDH; LDH; PDI; mRNA decapping protein and 2 hypothetical proteins) were identified. Interestingly, no specific antigen spots were recognized by sera on any of the *Besnoitia* species studied and a similar antigen profile has been observed for *B. tarandi* and *B. besnoiti* sera when cross-reactions were studied. This fact corroborates the difficulty in discerning *Besnoitia* infections using current serological assays. The present study underscores the importance of sequencing the *B. besnoiti* genome for species diversity studies of the genus *Besnoitia.*

Keywords: *Besnoitia besnoiti; Besnoitia tarandi;* Difference gel electrophoresis (DIGE); Mass spectrometry; Immunome; Proteome.

1. Introduction

Besnoitia besnoiti and *B. tarandi* are two cyst forming apicomplexan parasites that belong to the genus *Besnoitia*, subfamily Toxoplasmatinae and family Sarcocystidae. This genus also includes other 8 species (*B. bennetti, B. jellisoni, B. wallacei, B. darlingi, B. caprae, B. akadoni, B. neotomofelis* and *B. oryctofelisi*) (Nganga et al., 1994; Dubey et al., 2002, 2003 a, c, 2004, 2005; Dubey and Lindsay, 2003; Oryan and Azizi, 2008; Dubey and Yabsley, 2010). However, only 4 of them (*B. besnoiti*, *B. bennetti, B. caprae*, and *B. tarandi*) infect ungulates (cattle, equids, goats and wild ruminants, respectively). *B. besnoiti* is the causative agent of bovine besnoitiosis (Marotel, 1912), an emerging disease in Europe (EFSA, 2010) that has also been reported in Africa, Asia and South America (reviewed by Olias et al., 2011). Two asexual and infective stages of this parasite develop in cattle, which act as the intermediate host. First, the fast-replicating tachyzoites multiply inside endothelial cells of blood vessels, producing the acute stage of the disease characterized by hyperthermia, photophobia, anasarca and orchitis. Afterwards, the slowly dividing bradyzoites produce the characteristic skin lesions and tissue cysts mainly located in conjunctiva, upper respiratory tract and distal genital track in females (reviewed by Álvarez-García et al., 2013).

Additionally, reindeer and caribou act as intermediate hosts for *B. tarandi*, which causes similar clinical signs as those caused by *B. besnoiti*, at least during the chronic phase of the disease. This phenomenon was first reported in reindeer (*Rangifer tarandus tarandus*) and caribou (*Rangifer tarandus caribou*) by Hadween (1922) in Alaska (USA) by microscopy, and similar infections have also been reported in reindeers from Finland (Dubey et al., 2004). In addition *Besnoitia* spp. infections have been also reported in wild ruminants from other countries such as Canada, Sweden, Russia and Spain (mule deer, musk ox and roe deer) (Levine, 1961; Choquette et al., 1967; Wobeser, 1976; Ayroud et al., 1995; Leighton and Gajadhar, 2001; Ducrocq et al., 2012; Gutiérrez-Expósito et al., 2012).

Many biological and epidemiological characteristics inherent to these *Besnoitia* species remain unknown. Moreover, these infections cannot be distinguished using currently available serological tools because of high cross-reactions among these parasites (Gutiérrez-Expósito et al., 2012). Therefore, the link between the sylvatic and domestic life cycles of these *Besnoitia* species still remains to be elucidated (Basso et al., 2011; Millán et al., 2012).

Traditionally, the taxonomy and classification of parasites has been based only on phenotypic characteristics such as morphology, ultrastructure, life cycles, and host specificity. Some biological and ultrastructural features show significant differences between *B. besnoiti* and *B. tarandi* (reviewed by Olias et al., 2011). When comparing *B. besnoiti* and *B. tarandi* bradyzoites, differences in the number and morphology of rhoptries were reported between these species (Dubey et al., 2003a, 2004). Additionally, the results obtained from a variety of experimental infections have demonstrated that gerbils and rabbits are refractive to *B. tarandi* infection but susceptible to *B. besnoiti* infection (Pols, 1960; Neuman, 1962; Bigalke, 1968; Shkap et al., 1987; Dubey et al., 2004; Basso et al., 2011). Experimental infections in bovines

have only been carried out using *B. besnoiti* (Bigalke, 1968; reviewed by Álvarez-García et al., 2014c). Although different intermediate hosts have been described for these species, the degree of host specificity still remains uncertain. In addition, molecular studies are necessary for determining the phylogenetic relationship among different parasites (Tenter et al., 2002). Molecular characterization studies have shown a close relationship among the *Besnoitia* species affecting ungulates (Kiehl et al., 2010), and the molecular comparison of *B. besnoiti* 18S and ITS1 RNA sequences with those available for *B. tarandi* (Schares et al., 2009) also revealed a high degree of identity (> 99%). So far, genotypic differentiation among isolates from both species is limited to microsatellite analysis (Madubata et al., 2012). In *B. besnoiti*, proteomic approaches have allowed the description of the proteome and the immunome of the tachyzoite stage (García-Lunar et al., 2013b) as well as proteins that are differentially expressed in different developmental stages (Fernández-García et al., 2013). Although the genome sequence is not yet available, proteomic differences could help to elucidate variations in the tachyzoite biology between these species. Thus, the aim of the present study was to compare the protein expression of *B. besnoiti* and *B. tarandi* tachyzoites by 2D-DIGE. Furthermore, their immunogenic profiles were compared by 2-DE immunoblot.

2. Materials and methods

2.1. Parasites

Tachyzoites from *B. besnoiti* (BbSp-1 isolate, obtained from a naturally infected cow in Spain) and *B. tarandi* (obtained from a naturally infected reindeer in Finland) were grown in a Marc-145 cell monolayer and were purified as described by Fernández-García et al. (2009a). Tachyzoites were counted using a Neubauer chamber, and parasite viability was determined using trypan blue staining. Next, tachyzoites were pelleted by centrifugation at 1,350 ×*g* for 10 min and stored at −80 ºC until use (Fernández-García et al., 2009a). For DIGE experiments, tachyzoites were separated into 5 batches of approximately 4×10^8 tachyzoites/batch for each parasite.

2.2. Tachyzoite extracts.

Protein extracts were prepared as previously described García-Lunar et al. (2013b). Approximately 4×10^8 frozen tachyzoites were disrupted in 150 µl of lysis buffer and subjected to 3 cycles of rapid freezing and thawing using liquid nitrogen. Tachyzoites were further disrupted by sonication for 15 min at 4 ºC in a water bath sonicator (Ultrasonics Ltd.).

For immunoblot, solubilization was aided by the subsequent addition of 150 µl of rehydration buffer and insoluble material was removed by centrifugation at 13,000 ×*g* for 30 min at 4 ºC. Protein concentrations of the resulting supernatants were determined using the Bradford method using bovine serum albumin as a standard (García-Lunar et al., 2013b).

For DIGE, samples were precipitated using the 2D-Clean up Kit (GE Healthcare) and resuspended in 75 µL of DIGE solution (10 mM Tris, 7 M urea, 2 M thiourea, 2% CHAPS) after disruption by sonication. Protein concentration was quantified using the Bradford (Bio-Rad) assay employing BSA as the calibration standard, and CyDye

labeling was performed as described by Fernández-García et al. (2013). Briefly, 50 µg of protein per sample was labeled with 400 pmol of Cy2, Cy3 or Cy5 fluorochromes dissolved in DMF (99.8%). Afterward, reactions were quenched with 1 µL of 10 mM lysine. All DIGE gels included an internal standard, which was prepared by pooling equal amounts of protein from each biological sample in the experiment and labeling with Cy2 dye.

2.3. DIGE

A total of 150 µg of protein containing the internal standard (Cy2-labeled) and *B. besnoiti* tachyzoite (Cy3-labeled for gels 1, 2 and 3, Cy5-labeled for gels 4 and 5) and *B. tarandi* tachyzoite extracts (Cy5-labeled for gels 1, 2 and 3, Cy3-labeled for gels 4 and 5) (Table 1) were mixed, and an equivalent volume of loading buffer was added (7 M urea, 2 M thiourea, 4% CHAPS and 2% IPGphor buffer 3-11). Samples were loaded into hydrated 24 cm non-linear, pH 3-11 IPG strips (GE Healthcare). IEF and second-dimension separation was performed as previously described Fernández-García et al. (2013). Briefly, IEF was performed using an Ettan IPGphor 3 unit (GE Healthcare) at 120 V for 1 h; 500 V for 2 h; 500-1,000 V for 2 h; 1,000-5,000 V for 6 h; 5,000 V for 10 h. Before second dimension separation, strips were equilibrated (6 M urea, 30% glycerol, v/v, 2% SDS, 100 mM Tris-HCl, pH 8.0) by first adding DTE (2%) and then adding iodoacetamide (2.5%). Strips were sealed with 12% polyacrylamide gels and proteins were separated (15 W/gel) in an Ettan Dalt Six unit (GE Healthcare).

Image visualization and the analysis of the different abundances of spots from both *Besnoitia* species were performed as previously described by Fernández-García et al. (2013). The extended data analysis (EDA) module in the DeCyder software v6.5 was used to perform hierarchical cluster analysis (HCA) and principal component analysis (PCA), including only the proteins that were significantly differentially abundant between *Besnoitia* species. The Euclidean method together with an average linkage was used to calculate the distances and linkage, respectively, for the HCA.

Table 1. DIGE experimental design.

[a] Fluorochromes labeling extracts. IE: Internal standard

DIGE	Gel	*B. tarandi*	*B. besnoiti*	IE
B. tarandi extracts *versus* *B. besnoiti* extracts	1	Cy5	Cy3	Cy2
	2	Cy5	Cy3	Cy2
	3	Cy5	Cy3	Cy2
	4	Cy3	Cy5	Cy2
	5	Cy3	Cy5	Cy2

2.4. 2-DE SDS-PAGE and immunoblotting

A total of 100 µg of protein extract from each *Besnoitia* species was applied to an Immobiline pH 3-10 NL DryStrip (17 cm) (Bio-Rad). Isoelectric focusing (IEF) and second dimension electrophoresis was performed (García-Lunar et al., 2013b), and after separation, gels were transferred to PVDF membranes (Immobilon TM-P, Millipore, Bedford, USA). Additionally one replicate for each species of Coomassie brilliant blue (CBB)-stained gels (Kang et al., 2002) were carried out using the same conditions described above and 400 µg of

each protein extract for the description of each proteome.

For 2-DE immunoblot, rabbit hyperimmune sera were produced against tachyzoites of *B. besnoiti* (n = 1: R1) and *B. tarandi* (n = 1: R2). Briefly, two-month-old N-Z albino rabbits raised in captivity were immunized intra-dermally with 10^8 tachyzoites resuspended in an equal volume of Freund's complete adjuvant (Sigma®) for the first injection and intramuscularly with 10^8 tachyzoites resuspended in Freund's incomplete adjuvant at 4, 8 and 12 weeks postprimary injection. Sera were collected 4 weeks after the last immunization.

Both *Besnoitia* spp. protein extracts were analyzed by immunoblot using both hyperimmune sera in order to describe their immunomes as well as to analyze cross-reactive antigens. The analysis was performed essentially as previously described by García-Lunar et al. (2013b). Briefly, after blocking and washing, the PVDF membranes were incubated with the primary antibody: anti-*B. besnoiti* tachyzoite or anti-*B. tarandi* tachyzoite hyperimmune serum diluted 1:1,000 and 1:750, respectively, according to their IFAT titer. Afterwards the membranes were incubated with a peroxidase-conjugated anti-rabbit IgG antibody (SIGMA) at a 1:160,000 dilution. Immunoblots were exposed for 1-5 min using the Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). AGFA CP1000 processor and AGFA films (Curix/RP2 Plus, 18 cm × 24 cm) were used for image acquisition.

Image capturing and matching was performed using the PDQuestTM (Bio-Rad) program. Spots that were present/absent in at least two replicate immunoblots were considered to be reproducibly present/absent (García-Lunar et al., 2013b). All immunoblots performed in the study were highly reproducible, as matches rates varied from 80 to 100%.

2.5. MS analysis (MS-MS/MS)

Differentially abundant spots were excised from DIGE gels for MS analysis. If insufficient protein amounts were extracted, spots were excised from preparative 2-DE gels ran with a total of 400 µg protein and visualized with an MS-compatible Coomassie blue staining. Prior to MS analysis, spots from the preparative gel were matched with those visualized in DIGE gels using DeCyder software for accurate spot correspondence (Gorg et al., 2000). Differentially abundant spots selected were in-gel reduced, alkylated and digested with trypsin for protein identification. For mass fingerprint (PMF) identification, monoisotopic peptide masses observed with a signal to noise greater than 10 were compared with the NCBI nr (24.02.2011: 13135398 sequences; 4494708239 residues), and ToxoDB release 7.1 (*Neospora caninum* (Nc-Liverpool isolate): 7082 sequences; 6037978 residues) (*T. gondii*: TgME-49: 7993 sequences; 5950736 residues and TgVEG: 7846 sequences; 5484338 residues) (Gajria et al., 2008) databases using the MASCOT algorithm v2.3 (https://www.matrixscience.com). For MS/MS sequencing analyses, suitable precursors selected and fragmented using CID (with atmospheric gas) with 1 kV ion reflector mode and precursor mass win-

dows of ±5 Da. The plate model and default calibration were optimized for MS/MS spectra processing. For protein identification, MASCOT was used to search through the Global Protein Server v. 3.6 from Applied Biosystems (García-Lunar et al., 2013b). Protein information such as biological process, molecular function (identified with GO terms) and pathways was obtained from the ToxoDB 7.1 database (Gajria et al., 2008).

3. Results and discussion

3.1. Differentially abundant proteins detected by DIGE analysis

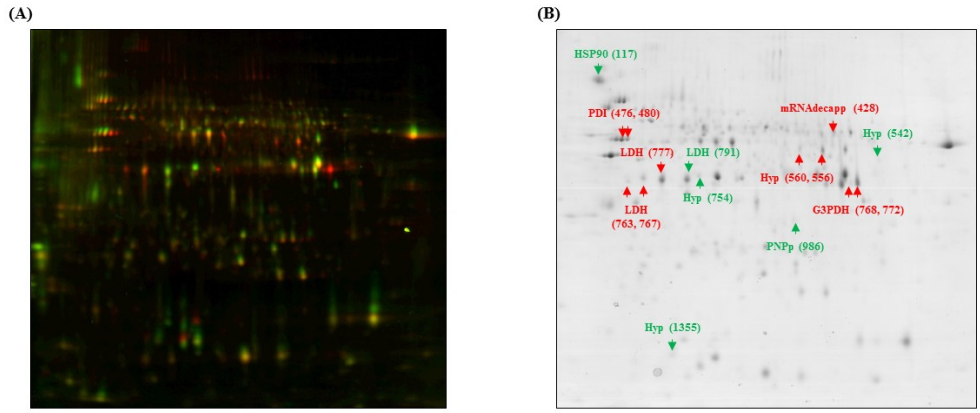
In this study, the species analyzed had similar proteome profiles with most of the spots located between 37 and 50 kDa and in the acidic range of the pH gradient (Fig. 1, Supplementary Fig. SF1). DIGE image analysis detected more than 1,400 spots on each gel, and spots with a significant increase (or decrease) in their relative abundance (±1.5-fold, $p < 0.05$, *t*-test) were considered to be differentially abundant. Bioinformatic analysis identified a total of 57 differentially abundant spots. Interestingly some of these spots were species-specific, and were only detected in one of the two proteomes. Both findings indicate the existence of variations in protein abundance between these two *Besnoitia* species (Fig. 1). Similarly, the hierarchical clustering analysis (HCA) and the principal component analysis (PCA) clearly distinguished *B. besnoiti* tachyzoites from *B. tarandi* tachyzoites (Fig. 2). Twenty-eight out of the 57 differentially expressed spots were more abundant in *B. besnoiti* tachyzoites, 4 of which could not be detected in *B. tarandi* tachyzoites.

However, the remaining 29 differentially expressed spots were more abundant in *B. tarandi* tachyzoites, and 13 of these were not found in *B. besnoiti* tachyzoites. It should also be considered that in this study, both parasites were cultivated under the same conditions and probably this might have contributed to reduce the differences between both species.

Few DIGE studies have been carried out comparing species, strains of organisms, and/or stages of the same parasite. However, using their results as definitive criteria for taxonomic purposes is difficult. Despite this fact, a proteomic approach can provide valuable data to discern the degree of divergence according to previous studies. Regidor-Cerrillo et al. (2012) compared three *N. caninum* isolates and showed different *in vitro* and *in vivo* behavior (Nc-Spain 7, Nc-Liv and Nc-Spain 1H) and detected 66 protein spots out of 2,500 with a significant increase or decrease in their relative abundance (±1.4-fold, $p < 0.05$, ANOVA and *t*-test). Moreover, Zhou et al. (2013) have recently compared type I, II and III strains of *T. gondii*, and 84 protein spots out of 2,321 were differentially abundant (±1.5-fold, $p < 0.05$, *t*-test). In a previous study on another protozoan, Davis et al. (2009) compared two species of *Entamoeba* and 141 spots out of 2,676 were more abundant in *E. histolytica* HM-1:IMSS than *E. dispar*, whereas 189 spots showed the opposite pattern. Contrary to our results, all of the previously mentioned studies did not report specific proteins for the extracts compared.

Fig. 1. DIGE gels of *B. besnoiti* and *B. tarandi* tachyzoite proteins.

(A): Overlay of images of gel 1 showing Cy3-labeled (*B. besnoiti* tachyzoite extract) and Cy5-labeled (*B. tarandi* tachyzoite extract) parasite proteins showing changes in the proteomes of *B. besnoiti* and *B. tarandi* tachyzoites. (B): Coomassie brilliant blue-stained DIGE gel. Proteins were separated in the first dimension along a non-linear pH gradient (pH 3-11, 24 cm; GE Healthcare) and on a 12% polyacrylamide gel in the second dimension. Identified proteins (Table 2) are indicated with arrows in green for spots that are more abundant in the tachyzoite stage of *B. besnoiti* and in red for spots that are more abundant in the tachyzoite stage of *B. tarandi*. Proteins are annotated with abbreviated names and master n◦ is shown in parentheses: HSP90: heat shock protein 90; Hyp: hypothetical protein; LDH: lactate dehydrogenase; PNPp: purine nucleoside phosphorylase, putative for *B. besnoiti* and mRNA decapp prot: mRNA decapping protein, putative; Hyp: hypothetical protein; LDH: lactate dehydrogenase; G3PDH: glyceraldehyde-3-phosphate dehydrogenase for *B. tarandi.*



The importance of these species-specific spots in *Besnoitia* biology still remains to be clarified. In addition, it remains to establish the influence of potential variations in protein expression intra-specie on the proteome differences between *B. besnoiti* and *B. tarandi* revealed in this study. In contrast to these results, a total of up to 262 differentially abundant proteins were obtained when *B. besnoiti* tachyzoites and bradyzoites were compared (Fernández-García et al., 2013). In this case, the different nature of the zoite origin (bradyzoites in an intermediate host *versus* tachyzoites in cell culture) may explain the higher number of stage-regulated proteins detected

3.2. Protein identification

As expected, the results obtained do not show any significant difference regarding functional processes between *B. besnoiti* and *B. tarandi*. This finding is most likely due to the similarity in terms of biologic activity between both species as well as among other Toxoplasmatinae protozoa. A total of 32 differentially abundant spots were excised from DIGE or preparative gels for MS analysis, but only 16 were successfully identified (6 spots were more abundant in *B. besnoiti* tachyzoites and 10 spots were more abundant in *B. tarandi* tachyzoites). By using mass fingerprinting, 14 spots were successfully identified, and MS/MS

combined with MS identified other 2 new spots. The identification of these 16 spots corresponded to 6 different proteins because different spots were recognized as the same protein, indicating that these protein species may involve multiple isoforms or post-translationally modified forms. In addition, 5 hypothetical proteins were also identified (Fig. 1, Table 2, Supplementary Table ST1). Although complete genomic information is not available for either of the *Besnoitia* species analyzed, at least 50% of the proteins were identified.

Fig. 2. (A) Hierarchical clustering of the 10 samples based on the global expression pattern of selected spots. The dendrogram on top shows the degree of similarity in protein abundance patterns between both *Besnoitia* extracts which are represented in columns. The dendrogram of individual proteins is shown on the left, with relative expression values displayed as heat map using a standardized log abundance scale ranging from –0.1 (down-regulated; green) to +0.1 (up-regulated; red). The gel number and dye labeling for each sample are listed below, and the spot number and protein identification are listed on the right-hand side.
(B) PCA analysis using the 57 selected proteins showing differences in abundance (±1.5-fold of change $p < 0.05$ using FDR) when the 5 replicates were compared. *B. besnoiti* replicates are shown in orange and *B. tarandi* replicates are shown in green. The proportion of variation in the dataset for each axis is indicated in parentheses.

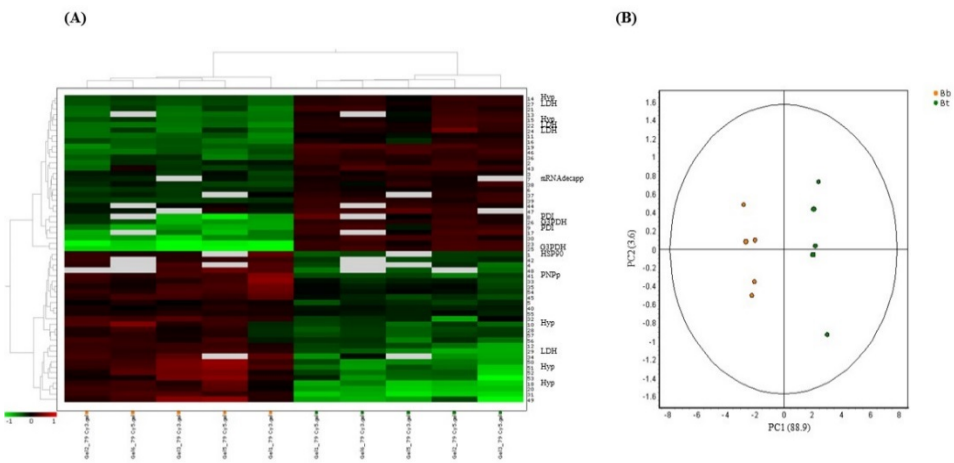


Table 2. Differentially abundant spots between *B. besnoiti* and *B. tarandi* identified by MALDI TOF MS and MS/MS analysis.

Master nº	Identified protein	Score	*B. tarandi vs B. besnoiti* comparison		Overabundant specie [a) b)]	Theo. MWS	Theo. p*I*	Nº matched peptides or amino acid sequence of peptides [c) d)]	Number of mass values searched	Sequence coverage (%) [e)]	Accession nº	Functional category [f) g)]
			T-test	Av. Ratio								
791	Lactate dehydrogenase (LDH)	174	0.002	-4.97	*B. besnoiti*	36095	6.03	19 [c)]	65	49%	gb|TGME49_032350	Glycolytic enzime [f)]
117	Heat shock protein 90, related (HSP90)	176	0.0085	-3.23	*B. besnoiti*	82113	4.99	26 [c)]	65	36%	psu|NCLIV_040880	Chaperone, stress response [g)]
986	Purine nucleoside phosphorylase, putative (PNPp)	68	0.0024	-4.15	*B. besnoiti*	33478	6.66	K.NIFLTPDGR.T (61) [d)]	-	7%	gb|TGVEG_094720	Nucleoside metabolic process [f)]
542	Hypothetical protein (Hyp)	53	0.00045	-7.91	*B. besnoiti* [a)]	50879	8.51	11 [c)]	65	22%	psu|NCLIV_004400	-
754	Conserved hypothetical protein (Hyp)	61	0.04	-3.56	*B. besnoiti*	112995	8.79	14 [c)]	65	18%	gb|TGVEG_062940	-
1355	Hypothetical protein (Hyp)	53	0.0022	-7.45	*B. besnoiti*	38328	6.41	8 [c)]	65	31%	gb|TGME49_012260	-
768	Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), related (G3PDH)	128	0.00012	18.64	*B. tarandi*	36795	6.83	-	-	9%	psu|NCLIV_041940	Glycolytic enzime [g)]
772	Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), related (G3PDH)	116	0.008	4.11	*B. tarandi*	36795	6.83	-	-	8%	psu|NCLIV_041940	Glycolytic enzime [g)]
763	Lactate dehydrogenase (LDH)	175	0.00012	4.4	*B. tarandi*	36095	6.03	19 [c)]	65	44%	gb|TGME49_032350	Glycolytic enzime [f)]

767	Chain A, *T. gondii* bradyzoite-specific LDH (LDH1) in complex with Nad and Oxq (LDH1)	138	0.011	3.45	*B. tarandi*	36047	6.06	16 [c]	65	41%	gi|310689950	Glycolytic enzime [g]
777	Lactate dehydrogenase (LDH)	146	0.00012	2.95	*B. tarandi*	36095	6.03	17 [c]	65	44%	gb|TGME49_032350	Glycolytic enzime [f]
476	Protein disulfide isomerase (*Besnoitia besnoiti*) (PDI)	106	0.047	5.28	*B. tarandi* [b]	53299	5.22	16 [c]	65	37%	gi|94962169	Cell redox homeostasis [g]
480	Protein disulfide isomerase (PDI)	78	0.001	6.33	*B. tarandi*	53222	5.18	15 [c]	65	26%	psu|NCLIV_011410	Cell redox homeostasis [g]
428	mRNA decapping protein, putative (mRNA decapp)	59	0.016	1.66	*B. tarandi* [b]	210627	8.82	R.TGDRWAGETAGIAEGAR.A (29) [d]	-	8%	psu|NCLIV_045720	RNA binding [g]
556	Hypothetical protein (Hyp)	77	0.0016	2.81	*B. tarandi*	45022	5.61	-	-	23%	psu|NCLIV_011270	-
560	Hypothetical protein (Hyp)	60	0.0036	3.24	*B. tarandi*	45022	5.61	-	-	25%	psu|NCLIV_011270	-

This table was made following the MIAPE guidelines developed by (HUPO-PSI) (Version 2.97.6, 10th February, 2012).

a) Proteins not found in *B. tarandi* proteome.

b) Proteins not found in *B. besnoiti* proteome.

c) Number of peptide mass values search/matched.

d) Amino acid sequence identified by MS/MS; the ion score is indicated in parentheses.

e) Amino acid sequence coverage for the proteins identified by MS and MS/MS.

f) Biological function (GO function).

g) Function described in previous studies.

As for previous proteomic studies carried out on *B. besnoiti*, most spots identified here are related to metabolism and, particularly, to the glycolysis process. In this work, two spots were identified as glyceraldehyde-3-phosphate dehydrogenase (G3PDH), and both of them were specifically detected in *B. tarandi* tachyzoites. Moreover, lactate dehydrogenase (LHD) was identified in 4 different spots, all showing similar molecular weight and p*I*, in accordance with other *Besnoitia* studies (García-Lunar et al., 2013b). However, the abundance of these spots varied between the *Besnoitia* species: one LDH spot was more abundant in *B. besnoiti*, whereas the other three spots were more abundant in *B. tarandi* (Figs. 1 and 2A, Tables 2 and ST1).

Moreover, heat shock protein 90 (HSP90) was identified in one spot that was more abundant in *B. besnoiti* tachyzoites (Figs. 1 and 2A, Table 2). Although expression of the HSP family has been observed during stress conditions, such as the differentiation process from tachyzoites to bradyzoites, its presence in the tachyzoite stage has also been described for *B. besnoiti*, *N. caninum* and *T. gondii* (Weiss et al., 1998; Marugán-Hernández et al., 2010; Zhang et al., 2011; García-Lunar et al., 2013b).

Interestingly, protein disulfide isomerase (PDI) was identified in two spots, both of which were more abundant in *B. tarandi* tachyzoites. Interestingly, one of them was detected only in this *Besnoitia* species (Figs. 1 and 2A, Table 2). PDI is the only *B. besnoiti* protein that has been described and characterized to date in tachyzoites (Marcelino et al., 2011), and it has been recently identified in the proteome of *B. besnoiti* tachyzoite stage showing similar MWS and p*I* as *N. caninum* and *T. gondii* (García-Lunar et al., 2013b). Similarly, Fernández-García et al. (2013) found this protein to be more abundant in the tachyzoite stage of *B. besnoiti*.

Finally, purine nucleoside phosphorylase and mRNA decapping protein have been identified herein for the first time appearing specifically in the *B. besnoiti* and in *B. tarandi* proteomes, respectively. Moreover, 1 out the 5 hypothetical proteins identified here was specifically detected in *B. besnoiti* tachyzoites.

Almost all the protein related to metabolism identified in this study and the two spots identified as PDI protein, which has been reported to play an important role in invasion in *T. gondii* and *N. caninum*, were more abundant in *B. tarandi* tachyzoites (Shin et al., 2004, 2005). Moreover, mRNA decapping protein, which is directly related to protein synthesis, was also found to be more abundant in this parasite species. Although previous studies have found multiple similarities between *B. besnoiti* and *B. tarandi*, our data indicate that related proteins with a high metabolic activity rate are more abundant in *B. tarandi* tachyzoites. This fact may be related to different mechanisms associated with virulence between both species (Davis et al., 2009). Therefore, to clarify this issue, further research on interspecies variability *in vitro* and *in vivo* should be carried out.

3.3. Immunome

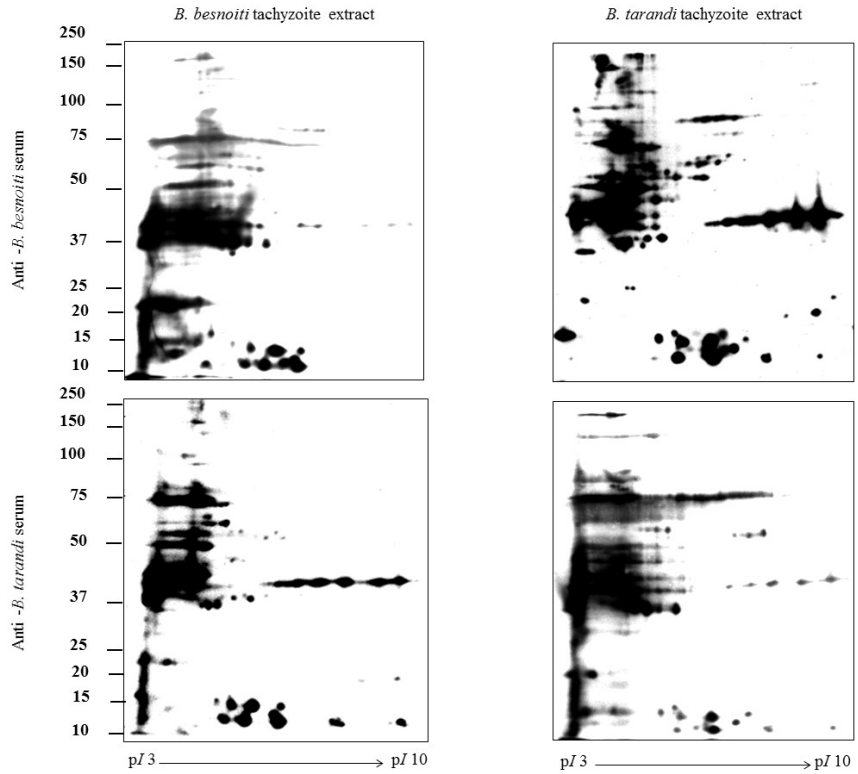
In the present work, approximately 92 ± 9, and 82 ± 8 spots were detected in *B. besnoiti* and *B. tarandi* 2-D immunome, respectively (Fig. 3). Most of the spots were

detected in the acidic range of the pH gradient and in three main antigenic areas: 75-50 kDa, 50-37 kDa, and 25-20 kDa (Fig. 3).

In this study, sera obtained from experimentally immunized rabbits were used and similar results compared to sera from natural infection have been obtained. Indeed, García-Lunar et al. (2013b) described approximately the same number of spots when the immunome of *B. besnoiti* tachyzoite stage was described by using sera from naturally infected cattle, and moreover, a similar antigen profile was shown, suggesting preservation of immunodominant antigens between host species. Related to this issue, Bjerkas et al. (1994) and Howe et al. (1998) previously showed that immune sera from a wide range of animal species either experimentally or naturally infected with *Neospora*, exhibited a similar recognition pattern when visualized by immunoblotting. However, novel immunogenic spots were identified herein found primarily between 10 and 20 kDa (Fig. 3). As expected, these antigens were not detected by sera from naturally infected cattle (García-Lunar et al., 2013b).

Fig. 3. Immunome profile of *B. besnoiti* and *B. tarandi* tachyzoite stage.

Protein extracts from both *Besnoitia* species were separated along a non-linear pH gradient (pH 3-10 NL IPG strips; 17 cm) in the first dimension and on a 10% polyacrylamide gel in the second dimension. Gels were transferred to PVDF membranes for the immunome and sera from experimentally immunized rabbits with *B. besnoiti* or *B. tarandi* tachyzoites were used. A replicate for each tachyzoite extract and rabbit serum is shown. The extract used is represented on top and the sera employed is shown on the left-hand side.



Although some spot chains (chain-like protein spots of equal molecular weight but different isoelectric points) appeared more intense in some cases, this fact was not related to the antigen and/or to the sera employed for the assays (Fig. 3). In fact, no specific antigen spots were recognized by sera on any of the *Besnoitia* species studied and the immunogenic profiles revealed for *B. tarandi* and *B. besnoiti* sera were similar when cross-reactions were studied (Fig. 3). These results are in accordance with a previous study where the same pattern of recognition was described for both *Besnoitia* species and serological cross-reactions were described for the first time by 1-DE Western blot (Gutiérrez-Expósito et al., 2012). Additionally, immunogenic spots in this study correlate with the 1-DE IDA pattern of the tachyzoite stage of *B. besnoiti* described by Fernández-García et al. (2009a), where six IDAs (14.2, 33, 37.1, 39.6, 46.3 and 190.8 kDa) were identified.

4. Conclusion

This study represents the first attempt to describe and quantify variations in the tachyzoite proteome of *B. besnoiti* and *B. tarandi* using proteomics, which identified differences in the protein abundance levels. The results could correlate with genetic differences still to be determined. This finding supports the hypothesis that *B. besnoiti* and *B. tarandi* might be different species. However, the differences observed here may be both specie-specific or strain-specific and further work should be done in order to clarify this issue. On the other hand, the results have also shown a conservation of the antigen repertoire between both *Besnoitia* species. This fact has not facilitated the identification of species-specific proteins useful for diagnostic purposes in this work. Therefore, the difficulty in elucidating the epidemiological gaps by current serological assays has been corroborated, and further work should be done to identify specific diagnostic targets. Moreover, due to the hydrophobicity and low abundance of membrane and secreted proteins, respectively, additional approaches are needed to overcome these obstacles and to identify valuable diagnostic targets. In addition, experimental infections in cattle and wild ruminants as well as invasion and proliferation assays are needed to determine the specific variability and to clarify the role of wild ruminants in *B. besnoiti* epidemiology. Finally, sequencing the *B. besnoiti* genome is necessary for inferring genetic differences among different organisms.

Conflict of interest

The authors have no conflict of interest.

Acknowledgements

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Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2014.09.003

Sub-objective 3.3

Development and characterization of monoclonal antibodies against *Besnoitia besnoiti* tachyzoites

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Running title: MABs against *B. besnoiti* tachyzoites.

Draft version of the manuscript.

Abstract

Almost no reagents exist for experimental investigation in *Besnoitia* spp. biology that may also be useful to develop new diagnostic assays. Thus, herein eight monoclonal antibodies (MABs) were obtained against a whole- and a membrane enriched- *B. besnoiti* tachyzoite extracts. Confocal lasser scanning microscopy and transmission electron microscopy studies were carried out to co-localize the recognized epitopes. Additionally genus- species- and stage-specificity were verified. Thus cross-reactions with the closely related protozoan *B. tarandi*, *Neospora caninum*, *Toxoplasma gondii* and *Sarcocystis* spp. together with the bradyzoite stage of *B. besnoiti* were investigated using Western blot. Results revealed that MABs 3.10.8 and 5.5.11 labeled the surface of *B. besnoiti* tachyzoites, MABs 1.17.8, 8.9.2 and 2.G.A recognized the apical tip of the tachyzoite and MABs 2.A.12, 2.F.3 and 2.G.4 recognized granular content inside the tachyzoite compatible with a dense granule staining. Accordingly, TEM results confirmed dense granule labeling with 2.F.3. Unexpectedly TEM results revealed that MAB 3.10.8 labeled dense granules. Remarkably, most MABs developed were genus-specific. Indeed none showed cross-reactions with *T. gondii* and only MAB 2.F.3 showed cross-reactions with *Sarcocystis* spp. Cross-reactions against *N. caninum* tachyzoites were only evaluated for MABs 2.G.A, 2.A.12, 2.F.3 and 2.G.4 and all showed a negative result. The absence of cross-reactions between MABs 1.17.8 and 2.G.A and *B. tarandi* supports their diagnostic value to differentiate *B. besnoiti* and *B. tarandi* infections. Finally, all MABs developed were *B. besnoiti* stage specific. This study has enhanced the tools available for *Besnoitia* cell biology with eight MABs. Ideally, MABs useful with diagnosis purposes requires the recognition of *B. besnoiti* specific and immunodominant antigens. Thus, the highly *B. besnoiti* tachyzoite specific MABs 2.G.A, 2.A.12 and 2.G.4 rise as promising diagnostic candidates. In addition, MABs labeling the surface of *B. besnoiti* tachyzoites may also be considered as potential targets. However, the identity of the antigens recognized by the MABs remains to be elucidated meanwhile *B. besnoiti* genome is not available.

Keywords: *B. besnoiti*; Tachyzoite; Monoclonal antibody; Confocal lasser scanning microscopy; Western blot; Transmission electron microscopy.

1. Introduction

Besnoitia besnoiti is a cyst forming apicomplexan parasite (Marotel, 1912) responsible for bovine besnoitiosis, a re-emerging disease in Europe characterized by both local and systemic clinical signs (reviewed by Álvarez-García et al., 2013). *B. besnoiti*, together with the closely related parasites *Toxoplasma gondii* and *Neospora caninum*, belongs to subfamily Toxoplasmatinae (Tenter et al., 2002). Members of this subfamily are characterized by the presence of two sequential asexual parasite stages that develop in the intermediate hosts. First, rapidly dividing tachyzoites are responsible for the acute stage of the disease, and later on tachyzoites switch into bradyzoites that develop inside tissue cyst during the chronic stage (reviewed by Álvarez-García et al., 2014c).

The morphology of *B. besnoiti* tachyzoites resembles to that described for the tachyzoite stage of *N. caninum* and *T. gondii*. Transmission electron microscopy (TEM) has revealed that *B. besnoiti* possesses typical Apicomplexan secretory organelles such as rhoptries, micronemes and dense granules, which may probably play an important role during its lytic cycle (Dubey et al., 2003a; Langenmayer et al., 2015b). Unfortunately, BbPDI which is thought to be secreted by *B. besnoiti* micronemes during host cell invasion as in *N. caninum* (Naguleswaran et al., 2005), is the only *B. besnoiti* protein identified and characterized to date (Marcelino et al., 2011). Thus, almost no reagents exist for experimental investigation in *Besnoitia* spp. A few proteomic studies have been carried out in order to investigate relevant biological processes as well as the host immune response associated with *B. besnoiti* infection (Fernández-García et al., 2013; García-Lunar et al., 2013b, 2014). However, the contribution of these studies has been hampered by the absence of *B. besnoiti* genome sequence. Surface antigens and proteins from secretory organelles, which have been described to be highly immunogenic in *N. caninum* and *T. gondii*, and therefore are considered good specific diagnostic targets (Saadatnia et al., 2012), were unable to be identified in *B. besnoiti* proteome (García-Lunar et al., 2013b). In this scenario, the development of MABs against specific *B. besnoiti* proteins seems to be a suitable approach considering that at least 25 *B. besnoiti* antigens cross-react with specific anti-*N. caninum* antibodies (García-Lunar et al., 2013b).

The aim of the present study was to develop new targets for *B. besnoiti* biology investigation and for diagnostic purposes. Thus, we have obtained and characterized eight monoclonal antibodies (MABs) directed against different compartments of *B. besnoiti* tachyzoite antigens. Confocal lasser scanning microscopy and TEM studies were carried out and specificity was also checked in the closely related protozoan *B. tarandi*, *N. caninum*, *T. gondii* and *Sarcocystis* spp. as well as in the bradyzoite stage of *B. besnoiti*.

2. Material and methods

2.1. Parasites

Tachyzoites from the BbSp-1 isolate of *B. besnoiti* (Fernández-García et al., 2009b), *B. tarandi* (Dubey et al., 2004), the Nc-1 isolate of *N. caninum* (Dubey et al., 1988) and the ME49 isolate of *T. gondii* (Lunde and Jacobs, 1963) were grown in a Marc-145 cell monolayer with DMEM

supplemented with 5% foetal calf serum and they were purified following a previously described procedure (Fernández-García et al., 2009b). Next, tachyzoites were pelleted by centrifugation at 1,350 ×*g* for 10 min and stored at -80 °C until use for Western blot assays (Fernández-García et al., 2009a).

B. besnoiti bradyzoites were released by trypsinization of a skin biopsy from a chronically naturally infected cow, following a previously described method (Fernández-García et al., 2009a). *Sarcocystis* spp. cystozoites were purified from naturally infected bovine hearts as previously described by Moré et al. (2008) and were pelleted by centrifugation and stored at -80 ºC until use for Western blot.

2.2. Production of hybridomas

MABs against both a whole *B. besnoiti* tachyzoite extract and a purified ELISA antigen (BbAPure) (Schares et al., 2013) were developed. Isotyping of MABs was performed with a commercially available kit (Sigma).

For the production of MABs against intact whole purified *B. besnoiti* tachyzoites BALB/C mice were immunized with 50 µg of extract 4 times separated by 15 days. Three days prior the fusion, mice were boosted by an intraperitoneal injection of 50 µg of extract. After euthanasia, splenocytes were fused with P3x63 Ag8653 myeloma cells. The supernatants of the hybridomas were screened for antibodies by ELISA. Positive hybridomas were cloned by limiting dilution and recloned at least 4 times.

MABs against BbAPure antigen were obtained following a previously reported procedure (Schares et al., 1999a; Aguado-Martínez et al., 2010). Briefly, mice were immunized by an intraperitoneal injection of 50 µg of *B. besnoiti* extract on day 0. Three days prior the fusion, mice were boosted by a subcutaneous injection of 50 µg of extract. Then, mice were euthanized and splenocytes were fused with SP2/0 myeloma cells. The supernatants of the resulting hybridomas were screened for antibodies against *B. besnoiti* tachyzoites by Western blot and positive hybridomas were cloned by limiting dilution and recloned at least twice.

All mice handling procedures complied with the EU legislation and were approved by the Ethics Commitee of the Complutense University of Madrid.

2.3. SDS-PAGE and Western blot

Coated membranes and immunoblot were performed following a previously described method in 15% polyacrylamide gels (Fernández-García et al., 2009a; García-Lunar et al., 2013a, 2015). A total of 4×10^7 tachyzoites from *B. besnoiti* and *B. tarandi,* 2×10^7 tachyzoites of *N. caninum* and *T. gondii* and 10^7 cystozoites from *Sarcocystis* spp, were used as antigen. Both, reducing and non-reducing conditions were employed for each antigen. For reducing conditions dithiothreitol (DTT) at 1:5 dilution was added before sonication in an ultrasonic bath.

2.4. Immunofluorescence

Screening of hybridoma cell culture supernatants was carried out as described by Schares et al. (1999a) and Aguado-Martínez et al. (2010) with few modifications. Briefly, 13 mm-coverslips of monolayer Marc-145 culture infected with 5×10^4 tachyzoites from Bb-Sp1 isolate. Two methods of fixation were employed. Two percent paraformaldehyde in PBS (2% PFA-PBS)

and 2% paraformaldehyde-0.05% glutaraldehyde in PBS (2% PFA+0.05%GA-PBS) were used as fixatives for 10 to 30 min at room temperature (RT). After fixation coverslips were permeabilised with PBS containing 0.2% Triton X-100 (Merck Chemicals) for 20 min at RT and blocked for 15 min at RT with PBS containing 0.1% Triton X-100, 3% bovine serum albumin (Roche). Then, cultures were labeled with a polyclonal antiserum against *B. besnoiti* tachyzoites (Gutiérrez-Expósito et al., 2012) (1:1,000 dilution) to delineate tachyzoite surface and with the hybridomas supernatants (non-diluted) for 30 min at RT. Following three washes with PBS, coverslips were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1,000) and Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:1,000) (Invitrogen) for 30 min at RT. Parasite and host cell nuclei were 40,6-diamidino-2-phenylindole (DAPI)-labeled. Finally, coverslips were mounted on glass slides with ProLong® Gold antifade reagent (Molecular Probes) and sealed with clear nail polish. Single stacks of immunofluorescence stainings were captured with a Leica TCS-SPE confocal laser-scanning microscope (Leica Microsystems) in the Department of Biochemistry and Molecular Biology IV of the Complutense University (Madrid). Image processing was performed using the LAS AF (Leica Microsystems) and the ImageJ software (http://imagej.nih.gov/ij/).

2.5. TEM

B. besnoiti tachyzoite-infected Vero cells were grown in T25 tissue culture flasks. After a 72 h culture period, the medium was removed, cells were washed with 100 mM sodium cacodylate buffer, pH 7.2, and were fixed in cacodylate buffer containing 3% paraformaldehyde and 0.05% glutaraldehyde for 1 h at 20°C. The cell layer was removed with a cell scraper, washed in cacodylate buffer, centrifuged, and the pellet was resuspended in cacodylate buffer. Cells were then dehydrated by sequential washing in progressively increasing concentrations of pre-cooled (-20°C) ethanol (30-50-70-90-100%), and finally embedded in LR-White resin as previously described (Hemphill et al., 2004; Risco-Castillo et al., 2007). Infiltration of the resin was carried out over 3 days at -20°C, with one resin change/day. Finally, the specimen blocks were polymerized at 60°C over night. Sections of 80 nm thickness were prepared using a Reichert and Jung ultramicrotome, place onto formvar-carbon-coated nickel grids (Plano GmbH, Wetzlar, Germany), and air-dried.

For immunogold labeling, sections were on-grid-labelled in a moist chamber with monoclonal antibody culture supernatants that were used undiluted for 1 h at 20°C, followed by three washes in PBS, 10 min each. Goat anti-rabbit conjugated to 10 nm diameter gold particles (Amersham) was applied at a dilution of 1:5 in PBS-0.3% BSA as secondary antibody. After another 3 washes, 10 min each, grids were air dried and contrasted with uranyle acetate and lead citrate (Hemphill et al., 2004). Specimens were viewed on a Phillips 600 TEM operating at 60 kV.

3. Results and discussion

We have developed eight MABs that clearly co-localize different subcellular compartments of *B. besnoiti* tachyzoite stage. A few MABs were developed in the past against *B. besnoiti* tachyzoites (Shkap et

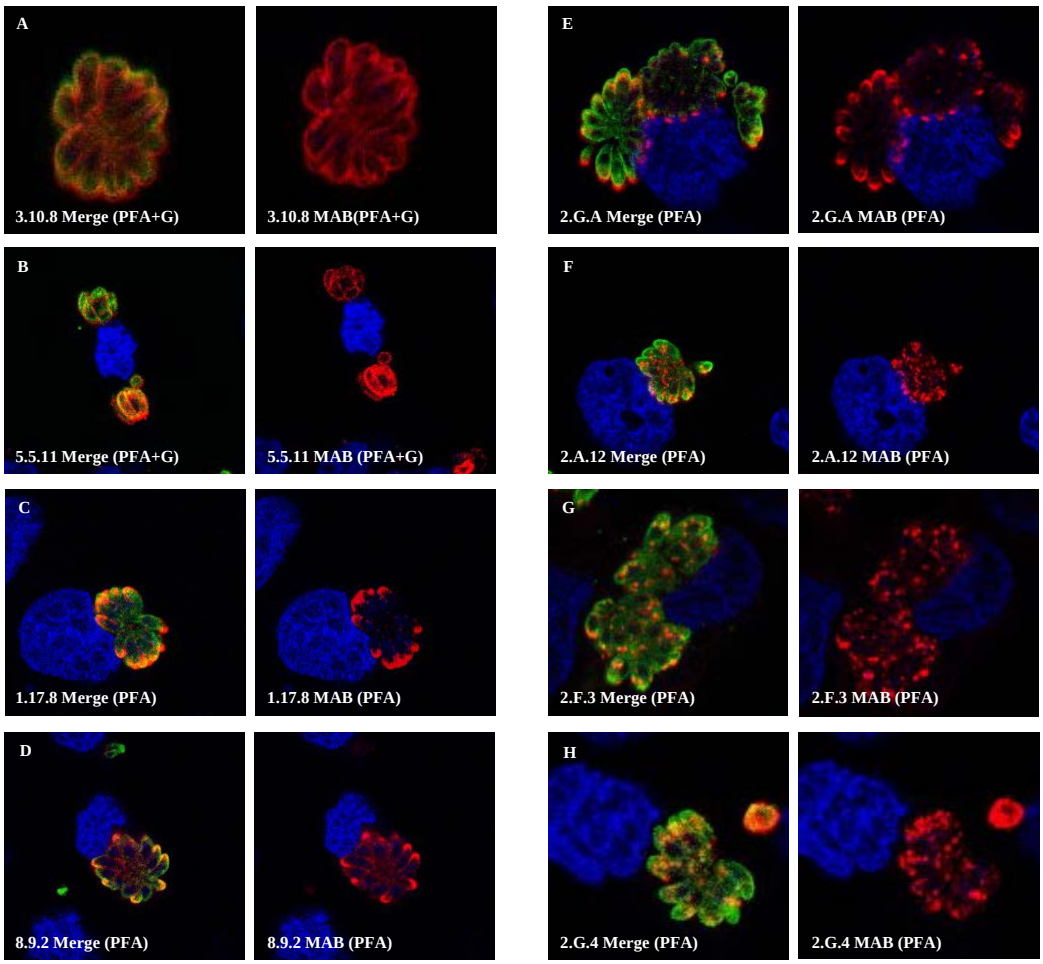
al., 1995; Njagi et al., 2004). However we have increased the number of MABs and have offered a more diverse panel of reagents in terms of localization of the epitopes recognized. Herein MABs were developed against a whole tachyzoite extract (designated as 2.G.A, 2.A.12, 2.F.3 and 2.G.4) and a membrane enriched extract (Schares et al., 2013) (named 3.10.8, 5.5.11, 1.17.8 and 8.9.2). The existence of a large number of important intracellular organelles characteristic of the members of the phylum Apicomplexa has been reported in *Besnoitia* spp. for which few probes are available. The characterization of these antibodies is timely since the production of MABs is essential for the development of new serological assays against bovine besnoitiosis as well as for the identification of *B. besnoiti* antigens involved in the lytic cycle. Similar approaches were successfully employed in related parasites (Sasai et al., 2008; Lamarque et al., 2012; Kang et al., 2015). In this sense, Baszler et al. (1996, 2001) developed and employed a MAB that recognized the surface protein NcSRS2 for the development of a competitive ELISA. Recently, Uzêda et al. (2013) employed a combination of MABs targeting a 38 kDa *N. caninum* tachyzoite surface protein (NcSRS2) and 33 kDa *N. caninum* dense granule protein (NcGRA7) to improve the immunohistochemical diagnosis of *N. caninum* infection. In addition, this approach allowed the investigation of biological features of this parasite since novel *N. caninum* secretory proteins with a key role in invasion including rhoptry bulb protein 2 (ROP2), apical membrane antigen 1 (AMA1) and rhoptry neck protein 4 (RON4) were identified (Sohn et al., 2011).

Co-localization studies using confocal lasser scanning microscopy have revealed that six MABs obtained in this study recognized intracellular content of *B. besnoiti* tachyzoites (1.17.8, 8.9.2, 2.G.A, 2.A.12, 2.F.3 and 2.G.4), whereas the other two remaining MABs labeled the surface of *B. besnoiti* tachyzoites (3.10.8 and 5.5.11) (Fig. 1). Interestingly, the later ones were produced against BbAPure antigen which is an enriched membrane antigen extract produced by surface byotinylation and subsequent immunoprecipitation (Schares et al., 2013). Schares et al. (1999a) obtained four MABs directed against biotinylated *N. caninum* tachyzoites and, similarly, three of them labeled the outer membrane surface of the parasite. On the other hand, MABs 1.17.8, 8.9.2 and 2.G.A clearly recognized the apical tip of the tachyzoite and MABs 2.A.12, 2.F.3 and 2.G.4 recognized granular content inside the tachyzoite compatible with a dense granule staining (Table 1).

Notably, MABs 1.17.8, 8.9.2, 2.G.A, 2.A.12, 2.F.3 and 2.G.4 were more clearly visible when PFA fixation was employed. On the contrary, visualization of *B. besnoiti* tachyzoites surface with MABs was improved using PFA+GA fixation. Others already observed these differences in antigen recognition depending on the fixation method employed. Similar to our results the MABs developed by Schares et al. (1999a) clearly recognized the surface of *N. caninum* tachyzoites using PFA+GA as fixative method. Moreover, Sohn et al. (2011) characterized rhoptries, dense granules and micronemes MABs by using PFA fixation.

Fig. 1. Confocal lasser scanning microscopy of MABs labeling the surface (A-B), the apical tip (C-E) and granular contain (F-H) of *B. besnoiti* tachyzoites.

Infected cultures were fixed with either PFA or PFA+GA and double labeled with anti-*B. besnoiti* policlonal antibody (green) and mouse MABs (red). Nuclei were stained with DAPI (blue). All the images show a single stack.



In accordance to confocal microscopy results, TEM results confirmed dense granule labelling with MAB 2.F.3 (Fig. 2). However, unexpectedly TEM results revealed that MAB 3.10.8 labeled dense granules contrary to the surface labeling observed by IFAT (Fig. 2). This finding might be a result of a dynamic colocalization of some proteins that may vary along the tachyzoite lytic cycle. This issue has been reported in *T. gondii* with *T. gondii* AMA1 protein that was released onto the surface of the tachyzoites from the micronemes. Similarly, at 1 h post-invasion, *N. caninum* microneme protein 3 (MIC3) protein was located on the surface of the tachyzoites, whilst at 3 h post-invasion NcMIC3 exhibited more a pronounced specific staining at the apical tip of the tachyzoite (Naguleswaran et al., 2001).

This is the first report on the development of *B. besnoiti* MABs directed against dense granules antigens. On the other hand, the MAB developed by Shkap et al. (1995) reacted against the apical complex. Similarly, Njagi et al. (2004) produced a panel of three MABs recognizing the epitopes associated with the anterior membrane and organelles of the apical complex and one MAB labeling a membrane-associated component.

Unfortunately, only Shkap et al. (1995) investigated the specificity of the MAB against a soluble fraction of *T. gondii* tachyzoites and a negative result was observed. However cross-reactions against *N. caninum* and *Sarcocystis* spp. were not studied. It has been addressed the need of verifying the specificity of new diagnostic targets for the development of specific diagnostic tools. In particular, *N. caninum* and *Sarcocystis* spp. infections are highly prevalent in cattle worldwide (Bartels et al., 2006; Moré et al., 2008; Eiras et al., 2011). Moreover anti-*N. caninum* and anti-*Sarcocystis* spp. specific antibodies cross-react with *B. besnoiti* causing false positive reactors (García-Lunar et al., 2015) and at least 25 *B. besnoiti* antigens conserved in eukaryotic cells cross-reacted with specific anti-*N. caninum* antibodies (García-Lunar et al., 2013b).

In order to verify the genus-, species- and stage- specificity of the MABs, cross-reactions with the closely related parasites *N. caninum*, *T. gondii*, *Sarcocystis* spp. and *B. tarandi* together with the bradyzoite stage of *B. besnoiti* were investigated using Western blot. The results are summarized in Table 1, together with the isotyping characterization. In general terms, the results of the present study revealed that most MABs developed in the present study were genus specific. None of the MAB showed cross-reactions with *T. gondii* either under reducing or non-reducing conditions. Unfortunately, cross-reactions against *N. caninum* tachyzoites could not be evaluated for MABs 3.10.8, 5.5.11, 1.17.8 and 8.9.2, due to the presence of cross-reactive antigens with antibodies present in the foetal calf serum employed for culture of hybridomas (Torres and Ortega, 2006).

Fig. 2. TEM images of MAB 3.10.8 (A) and 2.F.3 (B).
Reaction against dense granules of *B. besnoiti* tachyzoites is shown with arrows.

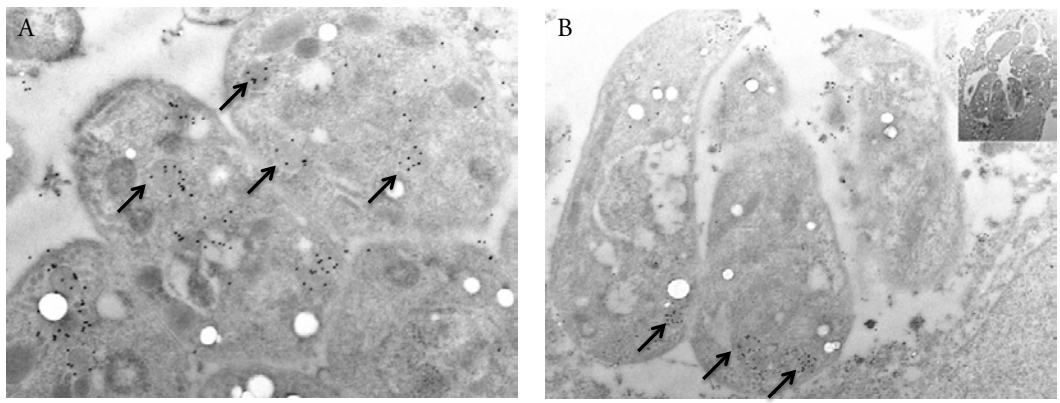


Table 1. Characterization of the MABs obtained against *B. besnoiti* tachyzoites: IFAT co-localization, immunoglobulin isotyping and protein bands detected by Western blot under reducing and non-reducing conditions.

MAB	Localization [a]	Isotyping	*B. besnoiti* T		*B. besnoiti* B		*B. tarandi* T		*N. caninum* T		*T. gondii* T		*Sarcocystis* spp. C	
			nr [b]	r	nr	r	nr	r	nr	r	nr	r	nr	r
3.10.8	Surface	IgG1	37.1 (35.1; 32.9)	- [c]	-	-	32.8; 30.3	-	ND [d]	ND	-	-	-	-
5.5.11	Surface	IgG1	19.7; 16.5 (22.3; 14.1)	-	-	-	19.9; 14.8 (18.3; 14.4)	-	ND	ND	-	-	-	-
1.17.8	Apical tip of the tachyzoite	IgG2a	224.3	-	-	-	-	-	ND	ND	-	-	-	-
8.9.2	Apical tip of the tachyzoite	IgG2a	42.8	-	-	-	39.8 (42)	-	ND	ND	-	-	-	-
2.G.A	Apical tip of the tachyzoite	IgG2b	84.5	-	-	-	-	-	-	-	-	-	-	-
2.A.12	Granular contain inside the tachyzoite	IgG2b	21.3 (23.1; 19.2)	20	-	-	19.4	20	-	-	-	-	-	-
2.F.3	Granular contain inside the tachyzoite	IgG1	29.2; 26.3	26	-	-	26.9	26	-	-	-	-	38.8	-
2.G.4	Granular contain inside the tachyzoite	IgG1	21.1 (23; 19.1)	20	-	-	19.4	19.4	-	-	-	-	-	-

T: tachyzoite; B: bradyzoite; C: cystozoite; nr: non-reducing conditions; r: reducing conditions.

[a] Localization of the protein band recognized by the MABs established by IFAT.

[b] Values in parenthesis indicate the molecular size of additional faint reactivity.

[c] No reactivity observed.

[d] No data available.

Notably, the remaining four MABs did not cross-react with *N. caninum*. Furthermore, only MAB 2.F.3 showed cross-reactions with *Sarcocystis* spp. cystozoite based Western blot under non-reducing conditions. In agreement, previous MABs developed against other Toxoplasmatinae members also showed cross-reactions with closely related parasites. In fact, the MAB directed against-a marker of *T. gondii* bradyzoites -TgBAG-1- cross-reacted with *N. caninum* bradyzoites (Weiss et al., 1999). Similarly the *T. gondii* bradyzoite-reacting CC2 MAB (Gross et al., 1996) was employed by Risco-Castillo et al. (2004) to confirm the maturation process of *N. caninum* cyst-like structures containing bradyzoites in an *in vitro* assay. Regarding specificity in genus *Besnoitia* most MABs, but for MABs 1.17.8 and 2.G.A, recognized *B. tarandi* antigens under both, reducing and non-reducing conditions.

These results agree with previous findings where *B. besnoiti* and *B. tarandi* infected animals showed the same pattern of immunodominant antigens and no specific antigenic spots for *B. besnoiti* and *B. tarandi* were recognized by 2-DE immunoblots (Gutiérrez-Expósito et al., 2012; García-Lunar et al., 2014). Thus, we corroborate the difficulty in developing specific tools to differentiate both infections. The species specificity of MABs 1.17.8 and 2.G.A should be further assessed with other *Besnoitia* species affecting ungulates such as *B. bennetti* and *B. caprae*.

All MABs were negative to *B. besnoiti* bradyzoites under both reducing and non-reducing conditions even though a similar proteome profile between both parasite stages has been described (Fernández-García et al., 2013). However, differences in the immunodominant antigenic (IDA) pattern between *B. besnoiti* tachyzoites and bradyzoites has been observed by others (Fernández-García et al., 2009a; Schares et al., 2010).

To conclude with, the approach followed here has enhanced the tools available for the study of *Besnoitia* cell biology with eight MABs. These MABs included two MABs recognizing the surface, three recognizing the apical tip and three recognizing a granular intracellular contain compatible with dense granules. Ideally, MABs with diagnostic value (E.g. to develop a competitive ELISA) should recognize *B. besnoiti* specific antigens. Thus, the highly *Besnoitia* spp. tachyzoite specific MABs 2.G.A, 2.A.12 and 2.G.4 together with those recognizing the apical tip of the tachyzoite (1.17.8 and 8.9.2), and the surface of the tachyzoites (3.10.8 and 5.5.11) rise as promising diagnostic candidates. Cross-reactions between MABs 3.10.8, 5.5.11, 1.17.8 and 8.9.2 with *N. caninum* should be first addressed. The absence of cross-reactions between MABs 1.17.8 and 2.G.A and *B. tarandi* supports their additional value as diagnostic markers to differentiate *B. besnoiti* and *B. tarandi* infections. Moreover, tachyzoite- specific MABs could be employed as markers of tachyzoite-bradyzoite conversion. The antigens recognized by the MABs developed herein should be identified which may be facilitated once the genome sequence is available. Finally, the potential role of these antigens recognized in the invasion process should also be elucidated.

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Sub-objective 3.4

A new ELISA test to diagnose *Besnoitia* spp. infection in bovids and wild ruminants renders unnecessary the use of a confirmatory test

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Abstract

Recent studies have reported that routinely used whole or soluble *Besnoitia besnoiti* tachyzoite (TZ) extract based ELISAs may raise a high number of false positive results with subsequent limitations in control and epidemiological studies of bovine besnoitiosis. Thus, Western blot (WB) has been recommended as a confirmatory test. In the present study, a new ELISA test that employs lyophilized tachyzoites (BbSALUVET ELISA 2.0) was developed and validated with cattle sera (n=606) under worst-case scenario. False positive and false negative soluble TZ extract based BbSALUVET ELISA 1.0 reactors were overrepresented and WB was considered as the reference test. One commercial test (PrioCHECK Besnoitia Ab 2.0 that employs whole TZ extract) and a recently developed membrane enriched ELISA (APure-BbELISA) were also tested. The three ELISAs showed high AUC values (> 0.9). However, the best diagnostic performance corresponded to BbSALUVET ELISA 2.0 and the APure-BbELISA [(92% sensitivity (Se); 98% specificity (Sp)] followed by PrioCHECK Besnoitia Ab 2.0 (88% Se; 98% Sp; 4.5% doubtful results). A different antigenic composition of lyophilized tachyzoites compared with whole or soluble tachyzoite extracts may be responsible for the improved diagnostic performance. In addition, BbSALUVET ELISA 2.0 was validated with wild ruminant sera, and better performance (96% Se; 97% Sp; 4% doubtful results) was obtained when compared with a previously developed BbSALUVET ELISA1.0 for *Cervidae* (100% Se; 86% Sp; 41% doubtful results). This study offers the use of BbSALUVET ELISA 2.0, which does not require a confirmatory WB in animals with ambiguous results, cattle prior to entry to herds free of the disease and valuable samples prior to a selective culling.

Keywords: *Besnoitia besnoiti*; Serodiagnosis; Cattle; Wild ruminants; BbSALUVET ELISA 2.0; PrioCHECK Besnoitia Ab 2.0, APure-BbELISA.

1. Introduction

Bovine besnoitiosis is a re-emergent disease of cattle caused by the cyst forming apicomplexan parasite *Besnoitia besnoiti*. At present, this disease continues spreading in Western Europe probably linked to management practices and animal trade with lack of surveillance that favors its dissemination (EFSA, 2010; reviewd by Álvarez-García et al., 2014c). Since there are no efficient treatments or vaccines, the control of bovine besnoitiosis entirely relies on accurate diagnosis coupled to herd management in order to detect both, acute and chronically infected animals with or without pathognomonic visible tissue cysts (Frey et al., 2013b; reviewed by Álvarez-García et al., 2014c). Thus, diagnostic assays are key tools for detecting subclinically infected animals and monitoring the success of control programs.

A wide number of serological techniques have been developed so far, and most of them have been validated in a large ring trial (García-Lunar et al., 2013a). The recommendations derived from this study included the usefulness of ELISAs for determining the serological status of a herd or conducting epidemiological studies, due to their easy performance and good diagnostic characteristics. However, two main limitations have been evidenced since then throughout several studies. First, the existence of *B. besnoiti* infected cattle with a false negative result has been reported (Fernández-García et al., 2010; Schares et al., 2010; García-Lunar et al., 2013a; Gutiérrez-Expósito et al., 2015). Second, recent studies have evidenced that specificity (Sp) is also compromised using not only ELISAs but also IFAT tests, which may cause a high proportion of false positive results (Schares et al., 2011a; Nasir et al., 2012; Gazzonis et al., 2014; Uzeda et al., 2014). To explain this last finding, the presence of cross-reacting antigens between *B. besnoiti*, *N. caninum* and *Sarcocystis* spp. was suggested, since a significant association has been found between *B. besnoiti* false positive ELISA results and the presence of *N. caninum* and/or *Sarcocystis* spp. antibodies (García-Lunar et al., 2015). Thus, the confirmation of an ELISA positive or negative result using Western blot (WB) test is recommended in particular situations such as inconclusive results, cattle prior to entry to herds free of the disease and valuable animals prior to a selective culling (García-Lunar et al., 2013a). Unfortunately, the availability of WB is usually limited to some specialized laboratories.

Thus, there is a need of developing more specific and at the same time sensitive ELISA assays for an accurate diagnosis. In addition, further comparative validation studies are essential for updating the diagnostic characteristics of the available tests. Besides, for diagnosis of bovine besnoitiosis, sera panels traditionally employed in validation studies should include a category of sera from cattle infected with *Sarcocystis* spp. and/or *N. caninum* with a *B. besnoiti* false positive ELISA result pointed out by García-Lunar et al. (2015) in order to standardize diagnostic assays under the worst-case scenario.

Ideally, new developed assays should be also adapted to detect *Besnoitia* spp. antibodies in other animal species including wild ruminants, since they may be useful tools for identifying potential wildlife reservoirs of the disease. So far, the only ELISA developed for this purpose showed

good diagnostic performance and *Besnoitia* spp. specific antibodies were successfully detected in Spanish wild ruminants (Gutiérrez-Expósito et al., 2013).

In the present study, a new ELISA test (BbSALUVET ELISA 2.0) was developed and validated with cattle sera following recent recommendations. For this purpose, false positive and false negative soluble tachyzoite- extract based (BbSALUVET ELISA 1.0) reactors were overrepresented and WB was considered as the reference test. One commercial test (PrioCHECK Besnoitia Ab 2.0) and a recently developed membrane enriched ELISA (Apure-BbELISA) were also re-validated. In addition, BbSALUVET ELISA 2.0 was also adapted to wild ruminant sera and diagnostic characteristics were estimated.

2. Material and methods

2.1. Sera

Two well-defined bovine and wild ruminant sera panels included the categories suggested by García-Lunar et al. (2013a, 2015) to validate BbSALUVET ELISA 2.0 and re-evaluate the other ELISA tests included in this study.

2.1.1. Bovine sera

A total of 606 sera were included and tachyzoite WB under non-reducing conditions was considered as the reference test. Sera were classified into four different categories according to an in house soluble tachyzoite- extract based ELISA (BbSALUVET ELISA 1.0) and WB results. All sera were analyzed using a lyophilized tachyzoite- extract based ELISA (BbSALUVET ELISA 2.0), a membrane enriched- extract based ELISA (APure-BbELISA) and one commercial test (PrioCHECK Besnoitia Ab 2.0).

Group 1: Sera from seronegative B. besnoiti non-infected cattle (n=250).

Sera from 214 cows and heifers from dairy and beef herds and 36 precolostral sera from calves from dairy herds were included in the study. A total of 151 sera came from herds with no history of bovine besnoitiosis, whereas the remaining 99 came from herds where the disease had been previously described. All sera were seronegative to *B. besnoiti* infection by both ELISA and WB (García-Lunar et al., 2013a).

Group 2: Sera from seropositive B. besnoiti infected cattle (n=216).

Sera came from cows and heifers from beef herds with a previous history of bovine besnoitiosis. No data regarding clinical signs were available for any of the animals sampled. However, all sera were seropositive to *B. besnoiti* infection by both ELISA and WB (García-Lunar et al., 2013a).

Group 3: Sera from BbSALUVET ELISA 1.0 false-positive reactors (n=105).

A total of 101 samples from adult cattle from both dairy and beef herds, and 4 precolostral samples from calves from dairy herds were included in the study. All sera proved to be positive to *B. besnoiti* infection by ELISA but proved to be negative by WB (García-Lunar et al., 2013a). Eighty-six samples were positive to both *N. caninum* and *Sarcocystis* spp. infections using tachyzoite- and cystozoite- based WB, respectively, whereas 7 and 12 samples were positive only to *N. caninum* or *Sarcocystis* spp. infections by ELISA and IFAT, respectively. (García-Lunar et al., 2015). In

particular, 2 out of the 98 sera positive to *Sarcocystis* spp. infection had *Besnoitia* IFAT titers of 1:50, 26 of 1:100, 43 of 1:200, 25 of 1:400 and 2 had titers of 1:800. Regarding anti-*N. caninum* antibody levels, 10 sera out of the 93 positive samples had RIPC values between 7-20, 28 between 21-40, 35 between 41-80 and 20 samples had RIPC values > 80 in the BbSALUVET ELISA 1.0.

Group 4: Sera from BbSALUVET ELISA 1.0 false-negative reactors (n=35).

A total of 35 sera came from adult cattle from endemically infected beef cattle herds that were studied over a 4-year period (2009-2013) (Gutiérrez-Expósito et al., 2015). Cattle included in the study were clinically inspected at the beginning and at the end of the study and macroscopic tissue cysts in the scleral conjuctivae and/or mucous membrane of the *vestibulum vaginae* were detected. Sera included in this study proved to be negative to *B. besnoiti* infection by ELISA but positive by WB (García-Lunar et al., 2013a).

2.1.2. Wild ruminants sera

A total of 326 sera from wild ruminants were included in the present work and tachyzoite WB under non-reducing conditions was considered as the reference test. Sera were classified in four different categories (groups 1, 2, 3 and 4) according to an in house soluble tachyzoite-extract based ELISA (BbSALUVET ELISA 1.0 for *Cervidae*) and WB (Gutiérrez-Expósito et al., 2013). In particular, category 4 was composed of sera showing doubtful results with BbSALUVET ELISA 1.0 for *Cervidae* since the conservative cut-off (negative values with RIPC < 16; positive values with RIPC > 37) established by Gutiérrez-Expósito et al. (2013) yielded a high number of doubtful results using the present sera panel. All sera were analyzed using BbSALUVET ELISA 2.0.

Group 1: Sera from seronegative non-infected wild ruminants (n=90).

Sera from 90 red deers from the South of Spain were included in the study. All sera were seronegative to *B. besnoiti* infection by both ELISA and WB (Gutiérrez-Expósito et al., 2013).

Group 2: Sera from seropositive Besnoitia spp. infected wild ruminants (n=88).

A total of 85 samples from naturally *B. tarandi* infected caribues (*Rangifer tarandus)* from Canada were included in the study. Additionally, 1 and 2 *Besnoitia* spp. infected red deer and roe deers, respectively, from Spain were also included. All sera were seropositive to *Besnoitia* spp. infection by both ELISA and WB (Gutiérrez-Expósito et al., 2012, manuscript in preparation).

Group 3: Sera from BbSALUVET ELISA 1.0 false-positive reactors (n=15).

A total of 11 and 4 samples from red deers and roe deers, respectively, from different areas of Spain were included in the study. All samples were seropositive by ELISA but proved to be negative by WB (Gutiérrez-Expósito et al., 2013, manuscript in preparation).

Group 4: Sera with a doubtful BbSALUVET ELISA 1.0 result either with a WB positive or negative result (n=133).

A total of 83 samples from red deers, 44 from roe deers and 6 caribues showing doubtful results were included in the study.

Only 7 samples, including 1 red deer, 1 roe deer and 5 caribues, proved to be positive by WB (Gutiérrez-Expósito et al., 2013, manuscript in preparation).

2.2. Parasites

B. besnoiti tachyzoites from Bb-Spain1 isolate (Fernández-García et al., 2009b) were grown in a Marc-145 cell monolayer with DMEM supplemented with 5% foetal calf serum and they were purified following a previously described procedure (Fernández-García et al., 2010). For BbSALUVET ELISA 1.0, tachyzoites were pelleted by centrifugation and stored at -80 ºC until used. For BbSALUVET ELISA 2.0, tachyzoites were preserved by lyophilization in a Virtis Benchtop K lyophilizator. Vials for lyophilization were prepared with 5×10^7 tachyzoites per vial and were resuspended in 4ml of PBS.

2.3. ELISA tests

BbSALUVET ELISA 2.0

Lyophilized tachyzoites were resuspended in coating buffer (0.1 M carbonate-bicarbonate, pH 9.6) and titrations of antigen (10^5, 5×10^5 or 10^6 tachyzoites/well) and conjugate (1:10,000 or 1:12,000) were performed. The combination offering the best discrimination between *B. besnoiti* infected and non-infected cattle was considered the optimum for the assay. The working conditions for the ELISA were determined using a short panel consisting of 20 bovine sera samples with a *B. besnoiti* positive, negative and false-positive BbSALUVET ELISA 1.0 result using WB as gold standard test. For the test, suitable positive- and negative-control sera were used (Fernández-García et al., 2010; Gutiérrez-Expósito et al., 2013). The optimized protocol is as follows. One hundred microlitres of coating buffer containing 5×10^5 lyophilized tachyzoites were added to each well of a polystyrene microtitre plate (Immuno Plate Maxisorp, Nunc, Roskilde, Denmark) and incubated overnight at 4 ºC. After three washes with phosphate-buffered saline containing 0.05% Tween 20 (PBST), blocking was performed with PBST containing 5% horse serum for 2 hours at room temperature (RT). Wells were then washed three times and were incubated with 100 µl of bovine or wild ruminant sera diluted 1:100 in blocking solution for 1 hour at 37 ºC. After three washes, 100 µl of a monoclonal antibovine IgG peroxidase-conjugated (Thermo Fisher Scientific) diluted 1/10,000 in PBST for bovine sera and 100 µl of a rabbit anti-deer IgG peroxidase conjugated (KLP, USA) diluted 1/800 in PBST for wild ruminant sera, were added to each well and was incubated 1 hour at 37 ºC. Wells were rinsed three times with PBST and bound antibodies were detected by incubation with 100 µl of 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic) acid substrate (Roche) at RT in the dark. After 10 minutes, the reaction was stopped by adding 100 µl of 0.3 M oxalic acid. Absorbance was measured as optical density values (OD) at 405 nm using a microplate reader (Multiscan RC 6.0, Labsystems). OD values were converted into a relative index percent (RIPC) by employing the following formula: RIPC = (OD405 sample - OD405 negative control)/ (OD405 positive control - OD405 negative control) x 100.

APure-BbELISA

ELISA was performed as previously described Schares et al. (2013) using a *B. besnoiti* tachyzoite membrane enriched extract as antigen. Sera showing PP values ≥ 1.754 were considered positive.

PrioCHECK Besnoitia Ab 2.0

A modified version of previously developed PrioCHECK Besnoitia Ab ELISA (Schares et al., 2011a) was re-evaluated following the manufacturer's instructions. Test results were expressed as sample/positive control (S/P) ratios based on positive- and negative-control sera. Sera ≤ 17 were considered as negative, between 17-23 as doubtful and ≥ 23% as positive.

2.4. Analysis of data

The precision of BbSALUVET ELISA 2.0 was measured by estimating intra-assay and 2 types of inter-assay repeatability (using the same and three different batches of antigen). Three replicates of 10 *B. besnoiti* positive and negative bovine sera were run in triplicate. Coefficients of variation (CV) [(standard deviation of the replicates/mean of replicates) x 100] were calculated using raw absorbance values. CV values less than 20% were indicative of adequate repeatability (Jacobson, 1998).

A non-parametric two-graph receiver operating characteristic (TG-ROC) analysis using SigmaPlot software was applied for the selection of cut-off values for the BbSALUVET ELISA 2.0 using either bovine or wild ruminants sera and to re-evaluate the other two ELISAs. Areas under the curves values (AUCs) were calculated for each assay and AUCs were compared using chi-square test (χ^2). A P value of < 0.05 was regarded as statistically significant. Afterwards, sensitivity (Se), Sp and test agreement (expressed as Kappa (k)-values), including 95% confidence intervals (95% CI), were calculated using WinEpiscope 2.0 (Thrusfield et al., 2001) and tachyzoite WB under non-reducing conditions was considered as the gold standard technique (García-Lunar et al., 2013a; Gutiérrez-Expósito et al., 2013).

3. Results

3.1. Precision

The mean CV values for BbSALUVET ELISA 2.0 were 3.1 (standard deviation (SD) mean value: 5.4), 4.7 (SD mean value: 0.029) and 9.4 (SD mean value: 9.4) for the intra-plate, inter-plate (using the same and three different batches of antigen) repeatability, respectively.

3.2. Diagnostic performance

All tests evaluated showed high AUC values (> 0.9) with the exception of the BbSALUVET ELISA 1.0 (AUC=0.87) (Fig. 1) and differences in AUC values were statistically significant only if the pair wise comparisons included the later (P < 0.05) (Fig. 1A).

Diagnostic characteristics and cut-off values are presented in Table 1. BbSALUVET ELISA 2.0 and APure-BbELISA showed excellent performance and only a few differences were observed between them (Fig. 2A, C). First, only 8 false-positive results were obtained with BbSALUVET ELISA 2.0 *versus* 5 false-positive reactors detected by APure-BbELISA out of 105 included. In addition, BbSALUVET ELISA 2.0 detected 14 false-negative reactors *versus* 20 detected with APure-BbELISA out of the 35 included.

Second, 216 samples from seropositive *B. besnoiti* infected cattle category were detected using BbSALUVET ELISA 2.0 *versus* 212 detected with APure-BbELISA out of 216 included. Finally, 249 samples out of 250 included from seronegative *B. besnoiti* non-infected cattle category showed negative BbSALUVET ELISA 2.0 result *versus* 247 showing negative APure-BbELISA result. Interestingly TG-ROC analysis of APure-BbELISA yielded a cut-off of PP > 2.07 for a slight increased Sp (98.9%) and equal Se values (92.5%).

On the other hand, PrioCHEK Besnoitia Ab 2.0 test showed high Sp (98.0%) since only 3 and 4 sera from false-positive reactors category showed a positive and a doubtful result, respectively. Moreover, 9 samples from seronegative *B. besnoiti* non-infected cattle showed doubtful results. However, lower Se (87.9%) was recorded due to several facts. First, 10 samples from seropositive infected cattle remained seronegative and 13 showed a doubtful result. In addition, 19 and 1 false-negative reactors showed negative and doubtful results, respectively.

Although TG-ROC did not improve Se values, a cut-off value of < 17.65 - 22.07 > can be employed to maintain the diagnostic characteristics and to decrease the number of doubtful results (Fig. 2D). As expected, BbSALUVET ELISA 1.0 showed the lowest Se and Sp values (86.1% and 70.4%, respectively) and no improvement of the test could be suggested after TG-ROC analysis (Fig. 2B).

Regarding serodiagnosis of *Besnoitia* spp. infection in wild ruminants, TG-ROC analysis displayed the same high AUC values for both ELISAs evaluated (p > 0.95) (Fig. 1B). BbSALUVET ELISA 2.0 showed high Se (95.7%) and Sp (96.8%) when a cut-off value < 31.5 - 35.7 > was applied. In this sense, only 2 samples out of the 15 false-positive reactors included showed also a false-positive result using the new test. Moreover, 3.9% of the samples analyzed showed doubtful results (Fig. 2E). Besides, perfect Se (100%) but lower Sp (85.7%) values were obtained when BbSALUVET ELISA 1.0 for *Cervidae* was re-evaluated. However, the conservative cut-off initially suggested remarkably yielded 40.7% of doubtful results. Accordingly, after TG-ROC analysis a cut-off value of < 31.4 - 50.6 > was suggested to keep good performance (95.6% and 97.2% Se and Sp respectively) with a substantial decrease of, doubtful results (6.4%) (Fig. 2F).

3.3. Test agreement

All ELISAs compared showed near perfect agreement (k > 0.8) with the exception of the pair-wise comparisons that included BbSALUVET ELISA 1.0, as this test showed the lowest Se and Sp values mentioned above (Table 2). BbSALUVET ELISA 1.0 and 2.0 for *Cervidae* also showed near perfect agreement (k > 0.8) and values improved after TG-ROC analysis (k > 0.9).

4. Discussion

A new ELISA (BbSALUVET ELISA 2.0) with improved performance compared to the traditionally employed serological techniques has been developed in the present study. Moreover, the diagnostic characteristics of the recently modified PrioCHECK Besnoitia Ab 2.0 ELISA test have been determined and APure-BbELISA was re-evaluated herein. Due to the re-emergence and spread of bovine besnoitiosis several epidemiological studies have been

Table 1. Se, Sp and doubtful results of the ELISA tests evaluated relative to tachyzoite WB under non-reducing conditions results before and after TG-ROC analysis using (A) bovine and (B) wild ruminant sera panel.

	Before TG-ROC analysis				After TG-ROC analysis			
(A)	Cut-off	Se (95% CI)	Sp (95% CI)	Doubtful results (%)	Cut-off	Se (95% CI)	Sp (95% CI)	Doubtful results (%)
BbSALUVET ELISA 2.0	-	-	-	-	RIPC > 17.34	91.7 (88.3-95.1)	97.5 (95.8-99.1)	-
BbSALUVET ELISA 1.0	RIPC > 9.7	86.1 (81.8-90.3)	70.4 (65.7-75.2)	-	-	-	-	-
APure-BbELISA	PP ≥ 1.756	92.4 (89.2-95.7)	97.7 (96.2-99.3)	-	PP > 2.07	92.5 (89.3-95.8)	98.9 (97.7-100)	-
PrioCHEK Besnoitia Ab 2.0	SP < 17- 23 >	87.9 (84.9-92.9)	98 (96.6-99.5)	4.5	SP < 17.65 – 22. 07 >	87.6 (83.4-96.1)	97.7 (96.1-99.3)	2.8
(B)								
BbSALUVET ELISA 2.0	-	-	-	-	< 31.5 – 35.7 >	95.7 (91.5-99.8)	96.8 (94.5-99.8)	3.9
BbSALUVET ELISA 1.0	< 16 – 37 >	100 (100-100)	85.7 (79.0–92.0)	40.7	< 31.4 – 50.6 >	95.6 (91.4-99.8)	97.2 (95.0-99.4)	6.4

recently carried out with PrioCHEK Besnoitia Ab 2.0 and BbSALUVET ELISA 1.0 (Nasir et al., 2012; Rinaldi et al., 2013; reviewed by Álvarez-García et al., 2014c; Ashmawy and Abu-Akkada, 2014; Gazzonis et al., 2014; Gutiérrez-Expósito et al., 2014; Papadopoulos et al., 2014).However, it has been recommended the use of a confirmatory specific WB since a high number of false-positive reactors may be detected by ELISA (Nasir et al., 2012; Gazzonis et al., 2014). This issue may notably compromise control programmes and surveillance. Besides, infected cattle may go under noticed by serological tests, as antibody levels may decrease below the detection limits of the assays in chronically infected animals (Fernández-García et al., 2010; Schares et al., 2010; Gutiérrez-Expósito et al., 2015). These animals represented a low percentage in outbreaks of the disease (1.11% and 3.2%) (Fernández-García et al., 2010 and Schares et al., 2010, respectively), respectively. However, it has been recently demonstrated that a higher number of false-negative reactors may appear in endemically infected herds (23%) (Gutiérrez-Expósito et al., 2015). This issue represents an important risk for disease spread, since new outbreaks are associated to animal trade from highly prevalent endemic areas into naïve herds (reviewed by Álvarez-García et al., 2013).

Fig. 1. TG-ROC analysis and AUC values showed by all ELISA relative to tachyzoite Western blot under non-reducing conditions results using bovine (A) and wild ruminant sera panel (B).

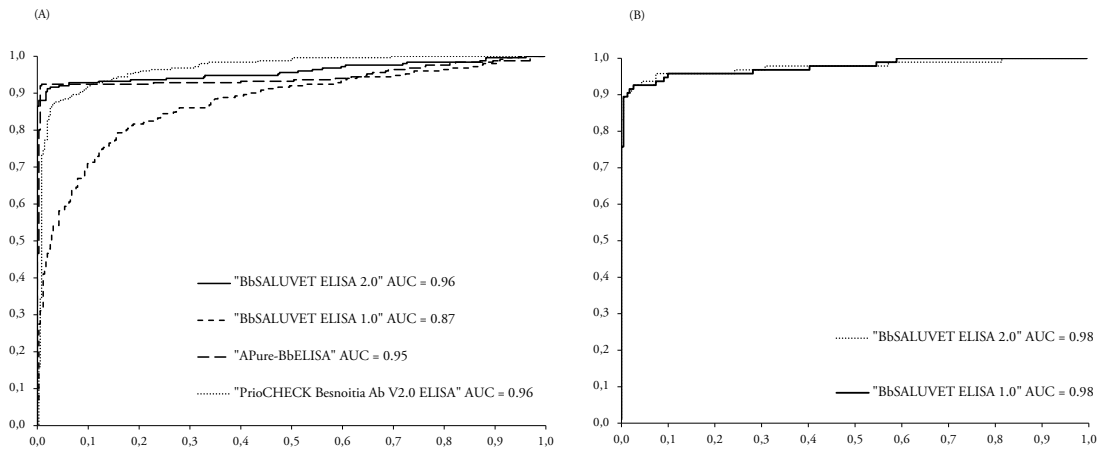

Table 2. Test agreement (*k*-values) between ELISA tests using bovine sera panel before TG-ROC analysis

* *K*-values after TG-ROC analysis are not presented since improved performance of the tests was not recorded after the analysis

	BbSALUVET ELISA 2.0	BbSALUVET ELISA 1.0	Apure-BbELISA	PrioCHEK Besnoitia Ab 2.0
BbSALUVET ELISA 2.0	1	0.635	0.917	0.879
BbSALUVET ELISA 1.0	0.635	1	0.586	0.566
APure-BbELISA	0.917	0.586	1	0.894
PrioCHEK Besnoitia Ab 2.0	0.879	0.566	0.894	1

Accordingly, the validation of BbSALUVET ELISA 2.0 was made under the worst-case scenario to overcome the above-mentioned limitations of serological diagnosis. Therefore, a high number of both, false-positive and false-negative reactors were included in the study apart from the traditionally employed categories composed of sera from seropositive infected and seronegative non-infected cattle. The analytical Sp has been estimated in different studies following OIE recommendations by analysing a few number of sera from cattle infected with other closely related apicomplexan parasites (*N. caninum, T. gondii, S. cruzi, S. hirsuta* and *S. hominis*) (Schares et al., 2011a, 2013; García-Lunar et al., 2013a). The results obtained in several studies including those reported in a European ring trial have suggested that a low number of these sera may cross-react with *B. besnoiti* antigens in serological assays. However, a recent study demonstrated why only some co-infected animals either with *Sarcocystis* spp. and/or *N. caninum* are *Besnoitia* false-positive reactors. It was revealed that *B. besnoiti* false-positive results are associated not only to the presence of *N. caninum* and *Sarcocystis* spp. co-infections, but also to high antibody levels against them (García-Lunar et al., 2015). Thus, a category of false-positive reactors that compiled these requirements was included in the present study.

In general terms, the three test evaluated in this study showed good performance. Notably, APure-BbELISA and BbSALUVET ELISA 2.0 have shown comparable results and both tests can be equally employed in diagnosis for control purposes. In particular, the excellent Sp of BbSALUVET ELISA 2.0 recorded, together with the good Se value observed, suggest the replacement of BbSALUVET ELISA 1.0 by this new assay. In addition, the excellent Sp previously reported for APure-BbELISA has been confirmed herein. This fact may probably be due to the appropriate sera panel employed for its initial standardization, which already included a high number of sera from cattle collected from herds showing *N. caninum*-associated abortions together with 10 samples showing false-positive and 10 showing borderline PrioCHECH Besnoitia Ab 2.0 results (Nasir et al., 2012). Furthermore, a notable improvement of Sp has been recorded using the new version of PrioCHECK Besnoitia

Ab 2.0 ELISA.The studies of validation carried out with this commercial kit throughout time have notably improved its performance. In fact, Schares et al. (2011a) evaluated the first version of this commercial test and 96.8% Sp was recorded at the cut off of 20% PP. However, when sera from cattle with *N. caninum* associated abortions were tested, positive ELISA results were associated to the presence of specific antibodies in the *N. caninum* immunoblot. Afterwards, Nasir et al. (2012) reported a high number of false-positive results using a second version of this commercial test (PrioCHECK Besnoitia Ab 2.0) at the cut-off of 15% PP. However, the more restrictive cut-off employed in the new version marketed (PrioCHECK Besnoitia Ab 2.0 ELISA) and evaluated here, together with the different control formulation, washing steps and results calculation also implemented, have notably improved the performance and 98% Sp has been recorded.

However, all tests evaluated showed Se values below our expectations, probably due to the moderate detection of sera from *B. besnoiti* infected cattle showing a false-negative BbSALUVET ELISA 1.0 result. Notably, PrioCHECK Besnoitia Ab 2.0 showed lower Se values than the other ELISAs tested, due also to the lack of detection of a few sera from seropositive *B. besnoiti* infected cattle category. Schares et al. (2011a) reported a similar observation using a first version of this test, particularly when reference-positive cattle without demonstrable tissue cysts were tested. At this point, subsequent validation studies have notably improved the performance of this test, and it is a difficult task to improve the Sp without a detriment of Se and *vice-versa*.

This work underscores not only the usefulness of re-evaluating serodiagnostic tests but also the necessity of up-dating the performance of serological assays to make results comparable. On the basis of the present results, the confirmation of ELISA positive results by *a posteriori* WB is no longer necessary since high Sp has been recorded for all the assays. Furthermore, several samplings throughout time together with an exhaustive clinical inspection may increase Se values of BbSALUVET ELISA 2.0 and APure-BbELISA, since chronically infected cattle may show fluctuations in the specific antibody levels (Gutiérrez-Expósito et al., 2015). Concerning PrioCHECK Besnoitia Ab 2.0, the employment of an additional WB test may be useful to avoid false negative results in valuable samples.

The additional usefulness of BbSALUVET ELISA 2.0 for detecting *Besnoitia* spp. antibodies in wild ruminants samples has been demonstrated here. Thus, it can be employed for epidemiological studies of bovine besnoitiosis and for investigating the link between the sylvatic and domestic life cycles of *Besnoitia* species affecting ungulates. Besides, more balanced Se and Sp values together with a substantial decrease of doubtful results were obtained using BbSALUVET ELISA 1.0 for *Cervidae* when the adjusted cut-off from the TG-ROC was applied.Contrary to the results obtained using cattle sera BbSALUVET ELISA 2.0 did not provide a significant improvement of the diagnostic characteristics when compared to BbSALUVET ELISA 1.0 for *Cervidae* results. This fact could be explained by the low percentage of false-positive ELISA results previously reported by Gutiérrez-Expósito et al. (2013), which may be

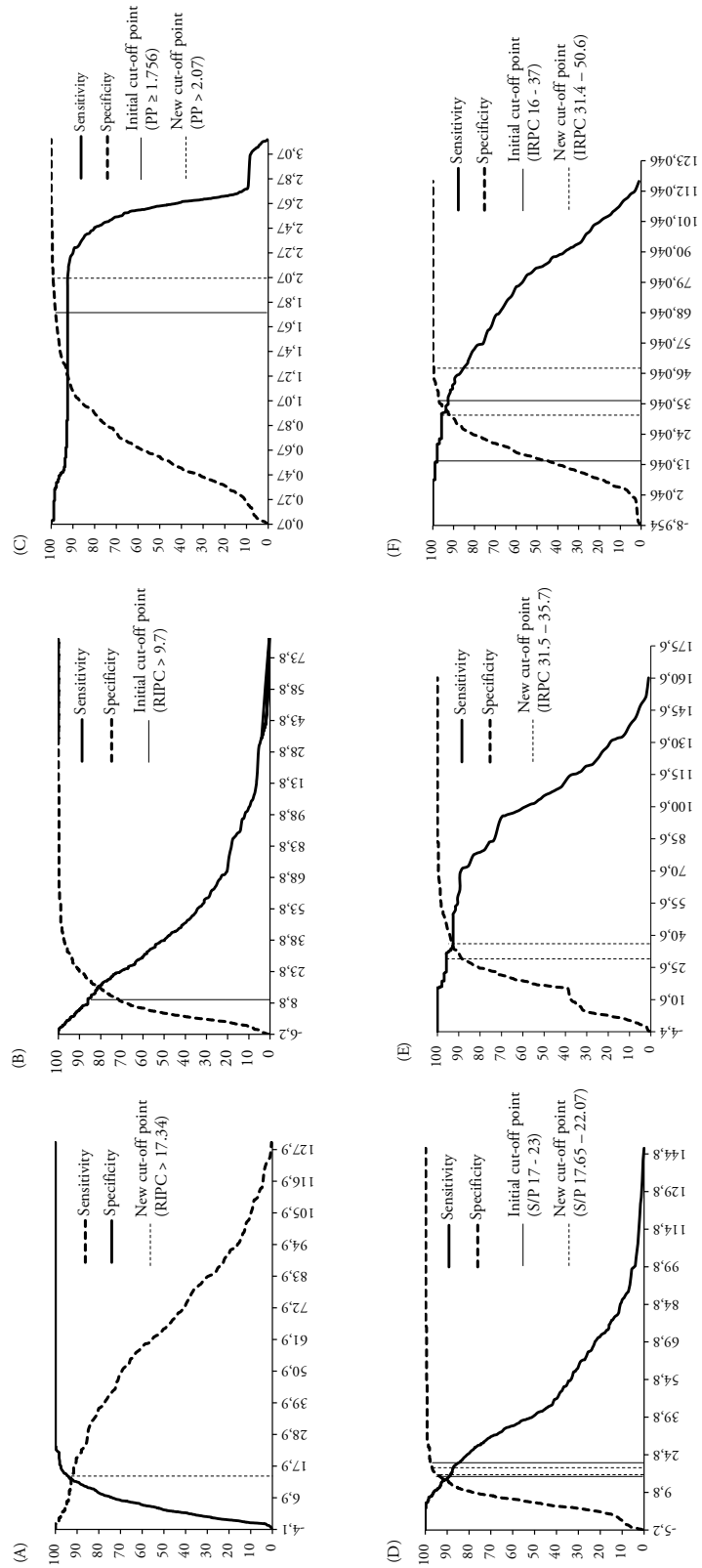

Figure 2. Se and Sp values showed by ELISA tests using bovine (A-D) and wild ruminants sera panel (E-F) and (A, E) BbSALUVET ELISA 2.0; (B, F) BbSALUVET ELISA 1.0; (C) APure-BbELISA and (D) PrioCHEK Besnoitia ELISA V2.0.

probably influenced by the low frequency of *N. caninum* and *Sarcocystis* spp co-infections and the low specific antibody levels against this parasites (García-Lunar et al., 2015). It is believed that approximately 100% of wild ruminants are infected with *Sarcocystis* spp. (Pérez-Creo et al., 2013), similarly to the reports made in cattle (Moré et al., 2008). However, the specific anti-*Sarcocystis* spp. antibody levels remain unknown, since seroprevalence studies have not been performed. Moreover, low *N. caninum* and *T. gondii* seroprevalence rates have been recently reported in roe deers and red deers from different areas of Spain (Gauss et al., 2006; Almería et al., 2007; Panadero et al., 2010).

The excellent performance observed for BbSALUVET ELISA 2.0 using either bovine or wild ruminant samples could be attibuted to the protein extract employed, which is based on lyophilized parasites. Comparable results have been obtained using APure-BbELISA where some of extract antigens employed were thought to be located in the surface of *B. besnoiti* tachyzoites, due to surface biotinylation and subsequent immunoprecipitation (Schares et al., 2013). Therefore, it is tempting to suggest an enrichment of membrane antigens in both extracts. Previous studies on *T. gondii* and *N. caninum* have reported the usefulness of surface antigens for a specific diagnosis. In contrast, soluble antigens usually localized in the cytosol may cross-react with other members of the Sarcocystidae family, and may interfere with serological diagnosis. In fact, up to 25 *B. besnoiti* cross-reacting antigens were recognized by a pool of sera from *N. caninum* infected cattle by 2-DE SDS-PAGE. A total of 5 spots could be identified and corresponded to highly conserved enzymes such as heat shock protein 60 and 90, fructose 1-6 bisphosphatase aldolase, enolase and actin (García-Lunar et al., 2013b). Based on these results, the lyophilization process may have contributed to the preservation of surface antigens whilst soluble cytosol antigens may have remained unexposed. To our knowledge, lyophilized parasites have been only employed for the detection of *T. gondii* infection in humans by IFAT (Takumi et al., 1966). However, comparative studies with the serological assays currently employed and based frequently on *T. gondii* surface and dense granules antigens have never been carried out (Aubert et al., 2000; Beghetto et al., 2003). Notably, apart from its easy scalable production, the good initial results of precision using different batches of antigen (which is supported by low CV values) makes it an attractive extract for future market exploitation. A similar approach that employed whole *N. caninum* tachyzoites fixed with formalin as antigen in ELISA showed good results (Williams et al., 1997). However, a later validation study evidenced lower Se values for this test (Von Blumröder et al., 2004). In this sense, the procedures employed for extraction and fixation of tachyzoites might interfere with the proper conformation of diagnostic epitopes required for a sensitive and specific diagnosis. Accordingly, improved performance of BbSALUVET ELISA 2.0 was not observed when *B. besnoiti* tachyzoites fixed either with formalin or ethanol were used as antigen in ELISA (data not shown).

In conclusion, a highly sensitive and specific ELISA has been developed in the present study and comparable results using

APure-BbELISA have been recorded. Therefore, both tests can be used indistinctly without the need of confirmatory assays. Moreover, PrioCHECK Besnoitia Ab 2.0 has been validated herein and excellent Sp has been recorded. However, WB should be still recommended to avoid false-negative results. In addition, the versatility of BbSALUVET ELISA 2.0 has been demonstrated due to its usefulness for the diagnosis of *Besnoitia* spp. infection in wild ruminants. Despite a similar antigenic composition of the extracts employed in both, BbSALUVET ELISA 2.0 and APure-BbELISA has been suggested, this issue remains to be further investigated together with the identification of specific antigens responsible of the improvement of diagnosis. Finally, based on the results of this study, a similar approach could be employed for improving the serological diagnosis of other protozoan infections.

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Capítulo V

Bovine besnoitiosis is a chronic and a debilitating disease of cattle characterized by both local and systemic manifestations of varying severity. Cattle may die during the course of the infection, although low mortality rates of less than 10% are expected. However, the disease is responsible for severe economic losses mainly due to a reduced value of the hides of affected animals, poor body condition, decreased milk production, abortion and transient infertility or even sterility in the male (reviewed by Álvarez-García et al., 2013).

During the last 20 years, several cases of bovine besnoitiosis have been recorded in cattle from Croatia, France, Germany, Hungary, Italy, Portugal, Spain and Switzerland (reviewed by Cortes et al., 2014). Therefore, due to the increased number of cases and the geographic dissemination of the disease into several countries, at present bovine besnoitiosis is considered to be re-emerging in Europe (EFSA, 2010). This fact is mainly associated with the entrance of the disease into naïve cattle herds by means of animal trade of unknown animal health status. Unfortunately, there are currently no efficient treatments or vaccines available and bovine besnoitiosis control relies solely on management measures coupled with diagnosis (clinical inspection and serological diagnosis) (reviewed by Álvarez-García et al., 2013). In this sense, several groups have developed a wide number of serological diagnostic assays to be employed for establishing adequate management measures for disease control. However, the standardization of the techniques developed so far is a crucial step for determining a gold standard test as well as common diagnostic procedures among affected countries. Moreover, comparable tests are urgently needed to carry out prevalence studies in different countries to determine the impact of the disease.

Unfortunately, recent studies have reported several limitations regarding serological diagnosis of bovine besnoitiosis. First, false-positive results may be detected using ELISA tests, which may notably compromise control programs and surveillance (Nasir et al., 2012; Gazzonis et al., 2014). Moreover, some chronically infected bovines showing tissue cysts may go unnoticed, since antibody levels may decrease below the detection limits of the tests (Schares et al., 2009; Fernández-García et al., 2010; Gutiérrez-Expósito et al.,

2015). In addition, *B. besnoiti* acutely infected cattle may not be detected since anti-*B. besnoiti* specific antibodies develop approximately after the second week post infection. Therefore, this is a limitation that is difficult solve. The employment of PCR and real time PCR assays (Cortes et al., 2007b; Schares et al., 2011b) for the detection of parasitemia during the early stages of the infection may be feasible options to overcome this limitation. Moreover, *B. besnoiti* and another *Besnoitia* species affecting wild ungulates (*B. tarandi)* are not differentiated by using these assays. In this sense, a previous study has reported the presence of anti-*Besnoitia* spp. specific antibodies in serum samples from red deers and roe deers from the Spanish Pyrenees (Gutiérrez-Expósito et al., 2013).

In this context, the present Doctoral Thesis covers three main objectives: *i)* the validation and standardization of the serological tests currently used in the diagnosis of bovine besnoitiosis (Objective 1); *ii)* the investigation of the origin of the false-positive results previously reported for the ELISA tests that are routinely employed in diagnosis and epidemiological studies (Objective 2) and *iii)* the identification of new diagnostic targets to improve the diagnostic characteristics of the available assays as well as for differentiating *B. besnoiti* and *B. tarandi* infections for epidemiological purposes (Sub-objectives 3.1, 3.2, 3.3 and 3.4). For this purpose, two different approaches were employed: first, proteomic studies were carried out to identify *B. besnoiti* specific and immunogenic proteins and MABs directed against *B. besnoiti* antigens were developed and characterized. Second, a *B. besnoiti* lyophilized whole tachyzoite extract was obtained and its usefulness for the development of a new ELISA test for the diagnosis of bovine besnoitiosis and *Besnoitia* spp. infections in wild ruminants was evaluated.

For the first objective (Objective 1), the serological tests available in Europe and routinely employed in four European laboratories were evaluated: SALUVET (Spain), Wusterhausen (Germany), Toulouse (France) and Bern (Switzerland). The tests evaluated were as follows: two IFATs, one in house ELISA (BbSALUVET ELISA 1.0), three commercial tests (INGEZIM BES 12.BES.K1 INGENASA, PrioCHECK Besnoitia Ab V2.0 and ID Screen Besnoitia indirect IDVET) and seven Western blot tests that were carried out using two extracts (tachyzoite- and bradyzoite- extracts) under

two different conditions (reducing and non-reducing) (Fernández-García et al., 2009a, 2010; Schares et al., 2010, 2011a; Liénard et al., 2011). Additionally, PrioCHECK Besnoitia Ab V2.0 ELISA was performed in all laboratories, and its reproducibility was evaluated. A well-characterized sera panel, which included a high number of sera from *B. besnoiti* chronically infected and non-infected cattle, was tested. Moreover, a few sera representative of *B. benoiti* acutely infected cattle together with a low number of sera from *T. gondii* and/or *N. caninum* infected bovines were also included.

In general terms, all tests evaluated in this study showed good performance, in particular when the "Majority of tests" gold standard was considered. Based on the results of the present study, any of the ELISAs evaluated could be used to determine the initial serological status of a herd due to their easy performance and good diagnostic characteristics. In particular, ID Screen Besnoitia indirect IDVET test and PrioCHECK ELISA 2.0 performed better than BbSALUVET ELISA 1.0 (SALUVET, Madrid) and INGEZIM BES 12.BES.K1 INGENASA. Unfortunately, PrioCHECH Besnoitia Ab V2.0 showed moderate interlaboratory precision since CV values showed noticeable differences among participants performing the test. In spite of this fact, these quantitative differences did not affect discrimination between seropositive and seronegative sera. Interestingly, there were no significant differences in Se between tachyzoite- and bradyzoite- based Western blots for the detection of acute cases of the disease and chronically infected animals, respectively. Related to this issue, a recent study has shown that both parasite stages share a high number of antigens (Fernández-García et al., 2013). Moreover, the bradyzoite specific proteins may remain unexposed to the immune system as an immune evasion mechanism. In particular, Western blots performed under non-reducing conditions and carried out by SALUVET-Madrid and FLI-Wusterhausen showed the highest Se and Sp values, regardless of the antigenic extract employed, and they should be used only to re-test animals with ambiguous results, cattle prior to entry to herds free of the disease and valuable animals prior to a selective culling. Considering cross-reactions, a low number of false-positive reactors was detected. However, a low number of sera from bovines infected with closely related parasites were included in the

study and Sp was certainly not accurately assessed. In this sense, only 16 out of 37 sera positive to *T. gondii* and/or *N. caninum* infections were positive by at least one of the techniques evaluated.

In this objective, the serological assays that were routinely employed for bovine besnoitiosis diagnosis in Europe were validated and standardized for the first time. Consequently, a common diagnostic procedure among affected countries could be recommended. This protocol included the usefulness of ELISA tests for determining the initial status of a herd and in epidemiological studies. However, Western blots are necessary to confirm ELISA results under the situations described above.

Despite the good initial results in terms of diagnostic characteristics derived from the first objective, more recent studies have reported the existence of false-positive results using PrioCHECK Besnoitia Ab2.0 and BbSALUVET ELISA 1.0 tests (Schares et al., 2010; Nassir et al., 2012; Gazzonis et al., 2014). Moreover, Uzêda et al. (2014) also reported the presence of *Besnoitia* false-positive IFAT results. It has been suggested that these false-positive reactions observed could be due to serological cross-reactions with related apicomplexan parasites (Schares et al., 2010; Nasir et al., 2012; Gazzonis et al., 2014). In particular, *Sarcocystis* spp. and *N. caninum* are two closely related parasites and both infections are highly prevalent in cattle worldwide (Dubey et al., 1989; reviewed by Dubey, 2003). Unfortunately, serological cross-reactions between *Sarcocystis* spp. that affect cattle (*S. cruzi, S. hirsuta, S. hominis* and *S. rommeli*) (reviewed by Dubey and Lindsay, 2006; Dubey et al., 2015) and *B. besnoiti* have never been studied. On the other hand, Shkap et al. (2002) have already reported that anti-*N. caninum* sera reacted with *B. besnoiti* antigens in two individual samples.

Consequently, the aim of the second objective (Objective 2) was to investigate the origin of the *B. besnoiti* false-positive ELISA results in order to establish a more appropriate panel sera to be employed in future validation assays. For this purpose, a high number of sera from *B. besnoiti* seronegative cattle with either a negative or a false-positive BbSALUVET ELISA 1.0 result together with *B. besnoiti* seropositive cattle was employed. Based on the results derived from Objective1, tachyzoite-based Western blot under non-

reducing conditions was considered as the gold standard test to classify sera as *B. besnoiti* seropositive or seronegative. Two additional categories, representative of closely related Sarcocystidae infections -*N. caninum* and *Sarcocystis* spp. infections-, were also included. Similar to *B. besnoiti* seropositivity criterion, Western blot was used as the definitive criteria to classify sera as *N. caninum* and *Sarcocystis* spp. seropositive or seronegative. In addition, IFAT and ELISA tests were employed to determine the anti-*Sarcocystis* spp. and anti-*N. caninum* antibody levels, respectively. Unfortunately, it is not yet clear whether Western blot or IFAT should be considered as the best gold reference test for the diagnosis of *Sarcocystis* spp. infection. In addition, Moré et al. (2008) described IFAT at low dilutions as a suitable method to diagnose *S. cruzi* infection in cattle. However, cross-reactivity at low IFAT titres has been widely described between other Sarcocystidae parasites (Shkap et al., 2002; Schares et al., 2010). On the other hand, Western blot has been recently considered as the reference technique for the diagnosis of *S. neurona* infection (reviewed by Howe et al., 2014). In this study, the pattern of *S. cruzi* cystozoite antigen recognition by Western blot was determined and those sera recognizing the 18-20 kDa antigenic area were considered *S. cruzi* seropositive. Whether the antigens described in the present study are shared antigens among other species of *Sarcocystis* needs future clarification.

In the present study, *Sarcocystis* spp. seropositive animals were more numerous than *N. caninum* seropositive animals. Remarkably the category of *B. besnoiti* seronegative animals with a false-positive ELISA result showed the highest number of sera with specific antibodies directed against both *Sarcocystis* spp. and *N. caninum* (74%), followed by the *N. caninum* seropositive animals category (52.8%). In contrast, *B. besnoiti* seronegative and seropositive infected animals hardly showed specific antibodies against both, *Sarcocystis* spp. or *N. caninum* (10.7% and 1.5%, respectively). As expected, the existence and *B. besnoiti* false-positive ELISA results was significantly associated with *N. caninum* and *Sarcocystis* spp. seropositivity.

Interestingly, those sera with a *B. besnoiti* false-positive ELISA result also showed significantly higher specific antibody levels against *Sarcocystis* spp. and/or *N. caninum*.

These results showed that *B. besnoiti* false-positive ELISA results are associated to both the presence and the level of anti-*Sarcocystis* spp. and anti-*N. caninum* specific antibodies. These results agree with those reported in the study carried out by Shkap et al. (2002), where cross-reactivity at low IFAT dilutions was only found between two sera showing high anti-*N. caninum* antibody levels (1:3,200) and *B. besnoiti* antigen (Shkap et al., 2002). Several *B. besnoiti* antigens responsible for these cross-reactions were recently identified by 2-DE SDS-PAGE throughout Sub-objective 3.1 of this Doctoral Thesis. In this study, up to 25 *B. besnoiti* cross-reacting antigens were recognized by pool sera from *N. caninum* infected cattle, and some of them were identified as highly conserved enzymes such as HSP60, HSP90, fructose-1,6-bisphosphate aldolase ENO and actin (García-Lunar et al., 2013b). Accordingly, when Schares et al. (2013) removed cross-reacting antigens with *N. caninum* during the development of an enriched membrane extract for ELISA, this new assay showed excellent performance.

In order to increase the Se and Sp of the serological assays for the diagnosis of *B. besnoiti* infection, new diagnostic targets for a sensitive and specific diagnosis were sought throughout Objective 3 of this Doctoral Thesis. For this purpose, first, the proteome and immunome of the tachyzoite stage of *B. besnoiti* were described by using 2-DE and the most abundant antigens were identified by MALDI-TOF/MS (Sub-objective 3.1). Moreover, cross-reactive antigens were also studied with cattle sera with *N. caninum* associated abortions, since, as demonstrated in Objective 2, anti-*N. caninum* specific antibodies cross-react with *B. besnoiti* antigens using ELISA.

Regarding the proteome of *B. besnoiti* tachyzoites, most spots were found between 37 and 50 kDa and in the acidic range of the pH gradient. Although previous studies in other Sarcocystidae parasites such as *T. gondii* and *N. caninum* (Cohen et al., 2002; Lee et al., 2003) are not comparable due to the different conditions employed, a similar spot distribution was observed, with a higher number of proteins located between 37 and 50 kDa and in the acidic range of the pH gradient. For describing the immunome of the tachyzoite stage of *B. besnoiti*, a pool of sera from naturally *B. besnoiti* infected cattle was used to avoid possible individual variations in the immune response as well as to identify

relevant antigens as diagnostic targets in natural infections. As for the proteome, and similar to the results found for *N. caninum* and *T. gondii* (Shin et al., 2005; Ma et al., 2009), most spots were located between 50 and 37 kDa and in the acidic range of the pH gradient. Interestingly, the distribution of immunogenic spots observed in this study, correlates with the 1-DE IDA pattern of the tachyzoite stage of *B. besnoiti* described by Fernández-García et al. (2009a), where six IDAs (14.2, 33, 37.1, 39.6, 46.3 and 190.8 kDa) were identified.

The most abundant spots were excised from CBB-stained gels for protein identification by MALDI-TOF/MS analysis. However, only 27 spots were successfully identified to be 20 different proteins, indicating the presence of multiple protein species, which may include isoforms, post-translational modifications and elements of protein processing. Only one of the 20 identified proteins corresponded to a specific *B. besnoiti* protein, BbPDI, which may be explained by the fact that *B. besnoiti* genome has not been sequenced yet and there are only a few *B. besnoiti* ESTs currently available. A study carried out on the molecular phylogeny of coccidian parasites has indicated that the *Besnoitia* genus belongs to Toxoplasmatinae subfamily (Ellis et al., 2000). Thus, successful identification of *B. besnoiti* specific proteins was achieved based on homology with sequences available in databases from other members of the Toxoplasmatinae subfamily, such as *N. caninum* (Nc-Liverpool isolate) or *T. gondii* (TgVEG and TgME49 strains).

Six out of 20 proteins identified in this study were related to energy metabolism, fructose-1,6-bisphosphate aldolase, putative phosphoglycerate kinase, LDH, pyruvate kinase, ENO 2 protein, and putative ATP synthase alpha chain. Moreover, three HSPs (HSP60, HSP70 and HSP90), four proteins involved in host cell invasion (actin, actin depolymerising factor, putative tubulin β chain and profilin family protein), and two proteins involved in cell redox homeostasis (thioredoxin-dependent peroxide reductase and PDI) were identified together with putative tryptophanyl tRNA synthetase, gbp1p, nucleoredoxin, putative receptor for activated C kinase and nuclear movement domain-containing protein. Due to the fact that all of the proteins identified in this study were conserved not only among apicomplexan parasites but also among eukaryote organisms,

cross-reactions with *N. caninum* sera samples were further investigated. Unfortunately, a total of 25 spots identified by immunoblotting with a pool of bovine sera from *N. caninum*-infected cattle were matched to *B. besnoiti* spots and five corresponded to previously identified proteins (fructose-1,6-bisphosphate aldolase, ENO, HSP60, HSP90, and actin). Interestingly, these cross-reacting protein spots, seen between *B. besnoiti* antigens and anti-*N. caninum* specific antibodies, and described in the present study, may be responsible for the serological cross-reactions previously observed in Objective 2.

Additionally, Sub-objective 3.2 focused on the comparison of the protein expression and the antigenic profiles of the two *Besnoitia* species affecting domestic (*B. besnoiti*) and wild (*B. tarandi*) ungulates by 2-DE DIGE and 2-DE immunoblot, respectively, in order to further identify *B. besnoiti* specific diagnostic targets and to determine the antigens responsible for the strong serological cross-reactions previously reported between both *Besnoitia* species (Gutiérrez-Expósito et al., 2012).

DIGE images together with bioinformatics analysis detected more than 1,400 spots in *B. besnoiti* and *B. tarandi* proteomes. A total of 57 spots were differentially abundant, which indicated the existence of variations between these two *Besnoitia* species. In particular, 28 out of the 57 differentially expressed spots were more abundant in *B. besnoiti* tachyzoites, four of which could not be detected in *B. tarandi* tachyzoites. On the other hand, the remaining 29 differentially expressed spots were more abundant in *B. tarandi* tachyzoites, and 13 of these were not found in *B. besnoiti* tachyzoites. In spite of this fact, in this study and similar to the results derived from Sub-objective 3.1, both species had similar proteome profiles with most of the spots located between 37 and 50 kDa and in the acidic range of the pH gradient. Several DIGE studies have been carried out comparing species (Davis et al., 2009), strains of organisms (Zhou et al., 2013), parasite isolates (Regidor-Cerrillo et al., 2012), and/or parasite stages (Marugán-Hernández et al., 2010; Fernández-García et al., 2013). Unfortunately, it is not possible to use their results as definitive criteria for taxonomic purposes. However, a proteomic

approach can provide valuable data to discern the degree of divergence according to previous studies.

As expected, the results obtained did not show any significant differentiation regarding functional processes between *B. besnoiti* and *B. tarandi*. This finding is most likely due to the fact that essential processes are shared between both species as well as among other Toxoplasmatinae protozoa. A total of 32 differentially abundant spots were excised from DIGE or preparative gels for MS analysis, but only 16 were successfully identified (six spots were more abundant in *B. besnoiti* tachyzoites and 10 spots were more abundant in *B. tarandi* tachyzoites). The identification of these 16 spots corresponded to six different proteins because different spots were recognized as the same protein, indicating that these protein species may involve multiple isoforms or post-translationally modified forms. In addition, five hypothetical proteins were also identified. As in Sub-objective 3.1, most spots identified here were related to metabolism and, particularly, to the glycolysis process. In this study, two spots were identified as GA3PDH, and both of them were specifically detected in *B. tarandi* tachyzoites. Moreover, LDH was identified in four different spots, all showing similar molecular weight and p*I*, in accordance with the results obtained in Sub-objective 3.1. However, the abundance of these spots varied between the *Besnoitia* species: one LDH spot was more abundant in *B. besnoiti,* whereas the other three spots were more abundant in *B. tarandi*. Moreover, HSP90 was identified in one spot that was more abundant in *B. besnoiti* tachyzoites. Although expression of the HSP family has been observed during stress conditions, such as the differentiation process from tachyzoites to bradyzoites, its presence in the tachyzoite stage has also been described for *B. besnoiti, N. caninum* and *T. gondii* (Weiss et al., 1998; Marugán-Hernández et al., 2010; Zhang et al., 2011). Intriguingly, PDI, previously reported by Marcelino et al. (2011), was identified in two spots, both of which were more abundant in *B. tarandi* tachyzoites. Interestingly, one of them was detected only in this *Besnoitia* species. Finally, purine nucleoside phosphorylase and mRNA decapping protein were identified herein for the first time appearing specifically in the *B. besnoiti* and *B. tarandi* proteomes,

respectively. Moreover, one out of the five hypothetical proteins identified here was only detected in *B. besnoiti* tachyzoites.

Almost all the proteins related to metabolism identified in this study and the two spots identified as PDI protein, which has been reported to play an important role in invasion in *T. gondii* and *N. caninum*, were more abundant in *B. tarandi* tachyzoites (Shin et al., 2004, 2005). Moreover, mRNA decapping protein, which is directly related to protein synthesis, was also found to be more abundant in this parasite species. Although previous studies have found multiple similarities between *B. besnoiti* and *B. tarandi*, our data indicate that related proteins with a high metabolic activity rate are more abundant in *B. tarandi* tachyzoites. This fact may be related to different mechanisms associated with virulence between both species (Davis et al., 2009). Therefore, to clarify this issue, further research on interspecies variability *in vitro* and *in vivo* should be carried out.

Regarding the description of the immunome, in the present study, most of the spots were detected in the acidic range of the pH gradient and in three main antigenic areas: 75-50 kDa, 50-37 kDa, and 25-20 kDa. In this study, sera obtained from experimentally immunized rabbits were used and similar results have been obtained compared to sera from natural infection. Indeed, approximately the same number of spots was detected when the immunome of *B. besnoiti* tachyzoite stage was described by using sera from naturally infected cattle and a similar antigen profile was shown, suggesting a preservation of immunodominant antigens between both species (Sub-objective 3.1).

Unfortunately, none of the proteomic studies carried out in this Doctoral Thesis have led to the identification of potential diagnostic targets, since all identified antigens were conserved proteins among apicomplexan parasites. However, this approach has allowed us to identify a high number of *Besnoitia* proteins for the first time, which included three proteins related to metabolism (fructose-1,6-bisphosphate aldolase, putative phosphoglycerate kinase, pyruvate kinase), two HSPs (HSP60 and HSP90), four proteins involved in host cell invasion (actin, actin depolymerising factor, putative tubulin β chain and profilin family protein) and one protein involved in cell redox homeostasis (thioredoxin-dependent peroxide reductase). Moreover, putative tryptophanyl tRNA

synthetase, mRNA decapping protein, purine nucleoside phosphorylase, gbp1p, nucleoredoxin, putative receptor for activated C kinase and nuclear movement domain-containing protein were also identified herein for the first time. Surface antigens and proteins from secretory organelles, which have been previously identified in other members of family Sarcocystidae, were not identified in any of the studies. As these proteins are highly immunogenic, they are considered good candidates for diagnosis (Saadatnia et al., 2012). However, due to the hydrophobic nature of membrane proteins and, probably, lower abundance of secreted proteins, additional approaches are needed to overcome these obstacles and to identify valuable targets (Bradley et al., 2005; reviewed by Bradley and Sibley, 2007; Marugán-Hernández et al., 2010; Che et al., 2011; Luo et al., 2011). Moreover, the failure in the identification of these proteins may be explained by the lack of the genome sequence of *B. besnoiti* together with the fact that genes encoding for proteins related to invasion may have diverged as for *N. caninum* and *T. gondii* (Reid et al., 2012).

For the identification of valuable diagnosis targets, MABs were additionally developed against a whole tachyzoite extract (designated as 2.G.A, 2.A.12, 2.F.3 and 2.G.4) and a membrane enriched extract (Schares et al., 2013) (named 3.10.8, 5.5.11, 1.17.8 and 8.9.2) (Sub-objective 3.3) and were characterized. In order to verify the genus- species- and stage- specificity of the MABs, cross-reactions with the closely related parasites *N. caninum, T. gondii, Sarcocystis* spp. and *B. tarandi* together with the bradyzoite stage of *B. besnoiti* were investigated, respectively, using Western blot.

Based on the results derived from co-localization studies, MABs 3.10.8 and 5.5.11 that were produced against an enriched membrane antigen extract, labeled the surface of *B. besnoiti* tachyzoites. On the other hand, MABs 1.17.8, 8.9.2 and 2.G.A clearly recognized the apical tip of the tachyzoite and MABs 2.A.12, 2.F.3 and 2.G.4 illustrated granular content inside the tachyzoite compatible with dense granule staining. TEM results confirmed dense granule labeling with MAB 2.F.3. Unexpectedly, TEM results revealed that MAB 3.10.8 labeled dense granules contrary to the surface labeling observed by IFAT. This finding might be a result of a dynamic co-localization of some proteins

that may vary along the tachyzoite lytic cycle. This issue has been reported in *T. gondii* with TgAMA1 protein as well as in *N. caninum* with NcMIC3 protein (Naguleswaran et al., 2001).

In general terms, the results of the present study revealed that most MABs were genus specific. Indeed, none of the MABs showed cross-reactions with *T. gondii* either under reducing or non-reducing conditions. Unfortunately, cross-reactions against *N. caninum* tachyzoites could not be evaluated for MABs 3.10.8, 5.5.11, 1.17.8 and 8.9.2, due to the presence of cross-reactive antigens with antibodies present in the foetal calf serum employed for culture of hybridomas (Torres and Ortega, 2006). Notably, the remaining four MABs did not cross-react with *N. caninum*. Furthermore, only MAB 2.F.3 showed cross-reactions with *Sarcocystis* spp. cystozoite based Western blot under non-reducing conditions.

Interestingly, most MABs, but for MABs 1.17.8 and 2.G.A, recognized *B. tarandi* antigens under both, reducing and non-reducing conditions. These results agree with findings reported in Sub-objective 3.2 of this Doctoral Thesis, where *B. besnoiti* and *B. tarandi* infected animals showed the same pattern of immunodominant antigens and no specific antigenic spots for *B. besnoiti* and *B. tarandi* were recognized by 2-DE immunoblots (García-Lunar et al., 2014). Thus, this study has corroborated the difficulty in developing specific tools to differentiate between the infections.

All MABs were negative to *B. besnoiti* bradyzoites under both reducing and non-reducing conditions even though a similar proteome profile between both parasite stages has been described (Fernández-García et al., 2013). However, differences in the immunodominant antigenic (IDA) pattern between *B. besnoiti* tachyzoites and bradyzoites have been observed by others (Fernández-García et al., 2009a; Schares et al., 2010).

Based on these results, the highly *Besnoitia* spp. tachyzoite specific MABs 2.G.A, 2.A.12 and 2.G.4, together with those recognizing the apical tip of the tachyzoite (1.17.8 and 8.9.2), and the surface of the tachyzoites (3.10.8 and 5.5.11) arise as promising

diagnostic candidates. However, cross-reactions between MABs 3.10.8, 5.5.11, 1.17.8 and 8.9.2 and *N. caninum* should first be addressed.

Finally, Sub-objective 3.4 of this Doctoral Thesis focused on the development of a new sensitive and specific ELISA test (BbSALUVET ELISA 2.0) that was validated using an appropriate sera panel, as stated in Objective 2 (García-Lunar et al., 2015). For this purpose, a new ELISA extract based on *B. besnoiti* lyophilized tachyzoites extract was obtained. Moreover, a recently developed tachyzoite membrane enriched ELISA (Apure-BbELISA) was also evaluated and the diagnostic characteristics of the recent version of PrioCHECK Besnoitia Ab 2.0 marketed were updated herein. In addition, the newly developed test was also adapted to detect *Besnoitia* spp. antibodies in wild ruminants, to determine its usefulness for further identifying *Besnoitia* infection in wild ruminants. For these purposes, the study was made under the worst-case scenario and a high number of both, false-positive and false-negative ELISA reactors were included in the study apart from the traditionally employed categories composed of sera from *B. besnoiti* seropositive and *B. besnoiti* seronegative cattle.

BbSALUVET ELISA 2.0 showed excellet Sp and good Se values, which suggested the replacement of BbSALUVET ELISA 1.0 by this new assay. Moreover, it showed good initial results of precision using different batches of antigen, as illustrated by the CV values. Consequently, the confirmation of ELISA positive results by *a posteriori* Western blot is no longer necessary. Notably, APure-BbELISA and BbSALUVET ELISA 2.0 showed comparable results and both tests can be equally employed in diagnosis for control purposes. In addition, the excellent Sp previously reported for APure-BbELISA was confirmed in this study. This fact may be due to the appropriate sera panel employed for its initial standardization, which already included a high number of sera from cattle showing *N. caninum*-associated abortions together with 10 samples showing false-positive reactions and 10 showing borderline results with PrioCHECH Besnoitia V2.0 (Nasir et al., 2012). Furthermore, a notable improvement of Sp was recorded using the new version of PrioCHECK Besnoitia Ab 2.0 ELISA. The studies of validation carried out with this commercial kit over time have notably improved its performance. Related to this issue,

BbSALUVET ELISA 1.0 was initially developed by Fernández-García et al. (2010) using a sera panel, which included a high number of *B. besnoiti* seropositive and seronegative samples and excellent Se and Sp was obtained. This test was later validated by García-Lunar et al. (2013a) during the first objective of the present Doctoral Thesis. Interestingly, the results demonstrated a minor decrease in both Se and Sp when a higher number of *B. besnoiti* seropositive and seronegative cattle sera were included. However, when a high number of false-positive reactors were included, as stated in Objective 2, several limitations were recorded.

On the other hand, all tests evaluated showed Se values below our expectations, probably due to the moderate detection of sera from *B. besnoiti* seropositive cattle showing a false-negative BbSALUVET ELISA 1.0 result. Based on this observation, several samplings over time together with an exhaustive clinical inspection may increase Se values of BbSALUVET ELISA 2.0 and APure-BbELISA, since chronically infected cattle may show fluctuations in antibody levels (Gutiérrez-Expósito et al., 2015). Notably, PrioCHECK Besnoitia Ab 2.0 showed lower Se values than the other ELISAs tested, due to the lack of detection of a number of sera from seropositive *B. besnoiti* cattle category. Schares et al. (2011a) reported a similar observation using a first version of this test, in particular when reference-positive cattle without demonstrable tissue cysts were tested. In this sense, concerning PrioCHECK Besnoitia Ab 2.0, the employment of an additional WB test may be useful to avoid false negative results in valuable samples.

The additional usefulness of BbSALUVET ELISA 2.0 for detecting *Besnoitia* spp. antibodies in wild ruminant samples has also been demonstrated here. Thus, it can be employed for epidemiological studies of bovine besnoitiosis and for investigating the link between the sylvatic and domestic life cycles of *Besnoitia* species affecting ungulates. Contrary to the results obtained using cattle sera BbSALUVET ELISA 2.0 did not provide a significant improvement in the diagnostic characteristics when compared to the results obtained using the only ELISA available for the diagnosis of *Besnoitia* spp. infection in wild ruminants (BbSALUVET ELISA 1.0 for *Cervidae*) (Gutiérrez-Expósito et al., 2013). This fact could be explained by the low percentage of false-positive ELISA

results previously reported by Gutiérrez-Expósito et al. (2013), which may be influenced by the low frequency of anti-*N. caninum* and *Sarcocystis* spp. antibodies and/or the low specific antibody levels against these parasites, according to the results observed in Objective 2.

The excellent performance observed for BbSALUVET ELISA 2.0 using either bovine or wild ruminant samples could be attributed to the extract employed, which is based on lyophilized parasites. Comparable results have been obtained using APure-BbELISA, where some of the antigens of the extract were thought to be located in the surface of *B. besnoiti* tachyzoites due to surface biotinylation and subsequent immunoprecipitation (Schares et al., 2013). Therefore, it is tempting to suggest a higher exposure to membrane antigens. Previous studies on *T. gondii* and *N. caninum* have reported the usefulness of surface antigens for a specific diagnosis (Schares et al., 1999b; reviewed by Montoya and Liesenfeld, 2004; Dubey and Schares, 2006; Petersen and Liesenfeld, 2007). In contrast, soluble antigens usually located in the cytosol may cross-react with other members of the family Sarcocystidae, and may interfere with serological diagnosis, as proved by the proteomic assay carried out in Sub-objective 3.1, where 25 cross-reacting antigens were recognized by a pool of sera from *N. caninum* infected cattle (García-Lunar et al., 2013b). To our knowledge, whole lyophilized parasites have never been employed before as ELISA antigen for the diagnosis of apicomplexan infections. Notably, the excellent performance observed, the easy and scalable production of the extract together with the good initial results of precision using different batches of antigen makes it an attractive extract for future market exploitation and the ELISA based on this extract has already been protected under intellectual property.

Capítulo VI

Objetivo 1. Comparación de las técnicas serológicas empleadas en el diagnóstico de la besnoitiosis bovina.

- **Primera.** Se ha establecido un protocolo de diagnóstico común tras la evaluación de las técnicas empleadas rutinariamente en el diagnóstico de la besnoitiosis bovina. En primer lugar, cualquiera de las pruebas ELISA evaluadas podría emplearse tanto en estudios epidemiológicos como en programas de control, gracias a sus buenas características diagnósticas y a su fácil ejecución. En segundo lugar, se considera el Western blot, basado tanto en el extracto del taquizoíto como del bradizoíto en condiciones no reductoras, como la prueba de referencia, al presentar los valores más elevados de Se y Esp. Su empleo se recomienda para confirmar el diagnóstico de la infección en el caso de animales con resultados dudosos, en las nuevas incorporaciones en rebaños libres de la infección y en animales valiosos antes de su sacrificio selectivo.
- **Segunda.** No se recomienda el empleo de las pruebas de IFI para el diagnóstico rutinario, ya que presentan el mayor número de resultados discordantes.

Objetivo 2. Investigación del origen de los resultados falsos-positivos en el ELISA para la detección de anticuerpos frente a la infección por *B. besnoiti*.

- **Primera.** Los resultados falsos-positivos en el ELISA están asociados no sólo a la presencia de anticuerpos específicos anti-*Sarcocystis* spp. y anti-*N. caninum,* sino también a un elevado nivel de anticuerpos frente a ambos parásitos. Este hecho explica por qué solo algunos animales seropositivos a *Sarcocystis* spp. y/o *N. caninum* dan lugar a resultados falsos-positivos mediante la prueba ELISA. Por ello, es necesario incluir un número apropiado de sueros procedentes de animales seropositivos frente a la infección por *Sarcocystis* spp. y *N. caninum* y que además presenten niveles elevados de anticuerpos frente a ambos parásitos en el proceso de validación de las técnicas serológicas de la besnoitiosis bovina.

Objetivo 3. Identificación de nuevas dianas diagnósticas en la infección por *B. besnoiti* y desarrollo de una nueva prueba serológica.

- Sub-objetivos 3.1 y 3.2
  - **Primera.** Se ha descrito, por primera vez, el proteoma y el inmunoma del estadio de taquizoíto de *B. besnoiti,* siendo similares, en ambos casos, a los de *B. tarandi.* Sin embargo existen variaciones en la abundancia de proteínas entre los taquizoítos de ambas especies.
  - **Segunda.** Existe un amplio número de antígenos conservados entre *B. besnoiti* y *B. tarandi*. En particular, se han identificado 17 proteínas abundantes y/o inmunogénicas en el proteoma de las dos especies, destacando la identificación de tres proteínas relacionadas con el metabolismo, dos HSPs, cuatro proteínas que participan en la invasión celular, una proteína que interviene en la homeostasis del potencial de óxido-reducción, junto con otras siete proteínas que participan en rutas conservadas. También se han identificado cinco proteínas responsables de las reacciones cruzadas con los anticuerpos específicos anti-*N. caninum*: fructosa 1,6 bisfosfatasa aldolasa, ENO, HSP60, HSP90 y actina.
  - **Tercera.** La complejidad para encontrar dianas diagnósticas de *B. besnoiti* mediante técnicas proteómicas, confirma la dificultad para diferenciar las infecciones producidas por *B. besnoiti* y *B. tarandi* mediante las técnicas serológicas disponibles en la actualidad. Por lo tanto, mientras el genoma de *B. besnoiti* no esté disponible, son necesarias otras aproximaciones que permitan identificar nuevas dianas diagnósticas.

- Sub-objetivo 3.3
  - **Primera.** Se ha obtenido un panel de ocho MABs, entre los cuales los MABs específicos del taquizoíto de *Besnoitia* spp. 2.G.A, 2.A.12 y 2.G.4 podrían ser buenos candidatos con fines diagnósticos, ya que no han mostrado reacciones cruzadas con otros miembros de la familia Sarcocystidae. Además, los MABs 3.10.8, 5.5.11, 1.17.8 y 8.9.2 también pueden considerarse buenas dianas

diagnósticas. Sin embargo, es esencial descartar la existencia de reacciones cruzadas entre estos MABs y *N. caninum*. Por otra parte, la ausencia de reacciones cruzadas entre los MABs 1.17.8 y 2.G.A y *B. tarandi* confirma su utilidad adicional como marcadores diagnósticos específicos.

- **Segunda.** Los MABs obtenidos constituyen nuevos reactivos de utilidad para el estudio de la biología de las especies de *Besnoitia*. Los MABs específicos de taquizoíto podrían emplearse como marcadores en el estudio de la conversión del estadio de taquizoíto a bradizoíto. Dos MABs han reconocido la superficie de los taquizoítos de *B. besnoiti*, tres han reconocido el extremo apical de los taquizoítos y tres han reconocido un contenido granular en el interior de los taquizoítos compatible con gránulos densos.

o Sub-objetivo 3.4

- **Primera.** Se ha desarrollado y validado una nueva prueba ELISA altamente sensible y específica basada en el empleo de taquizoítos liofilizados. La nueva prueba denominada BbSALUVET ELISA 2.0, presenta unas características diagnósticas similares a las obtenidas con la prueba APure-BbELISA, basada en el empleo de un extracto de taquizoíto enriquecido en proteínas de membrana. Ambas técnicas no precisan del empleo de pruebas diagnósticas confirmatorias. Por el contrario, la prueba PrioCHECK Besnoitia Ab2.0 ha mostrado valores moderados de Se y, por tanto, se recomienda el empleo de Western blot para confirmar los resultados.
- **Segunda.** Se ha demostrado la utilidad adicional de BbSALUVET ELISA 2.0 para el diagnóstico de la infección producida por *Besnoitia* spp. en rumiantes silvestres.

Objective 1. Comparison of the serological tools employed in the diagnosis of *B. besnoiti* infection in bovines.

- **First.** The results derived from this study have demonstrated that, for common diagnostic procedures, any of the ELISAs evaluated can be equally employed in epidemiological studies and control programs, due to their good diagnostic characteristics and easy performance. Additionally, tachyzoite- or bradyzoite-based Western blot tests performed under non-reducing conditions are considered to be the gold standard test since they have shown the highest Se and Sp values. Thus, they are recommended to re-test animals with ambiguous results, cattle prior to entry to herds free of the disease and valuable animals prior to a selective culling.
- **Second.** IFAT tests are not recommended for routinely diagnosis since they have shown the highest number of discordant results.

Objective 2. Investigation of the origin of *B. besnoiti* false-positive ELISA results.

- **First.** *Besnoitia besnoiti* false-positive ELISA results have been associated not only to the presence of anti-*Sarcocystis* spp. and anti-*N. caninum* specific antibodies, but also to high antibody levels against both parasites. This finding explains why only some seropositive animals either to *Sarcocystis* spp. and/or to *N. caninum* are *Besnoitia* false-positive reactors. In this sense, a high number of sera from cattle showing high antibody levels against either *Sarcocystis* spp. and/or *N. caninum* should be included in reference panel sera for an accurate validation of the serological assays.

Objective 3. Identification of new *B. besnoiti* diagnostic targets and development of a new serological assay.

- Sub-objectives 3.1 and 3.2
  - **First.** The proteome and the immunome of the tachyzoite stage of *B. besnoiti* have been described for the first time. Both, the proteome and immunome of *B. besnoiti* proved to be similar to the ones corresponding to *B. tarandi.* Despite these findings, differences in the protein abundance levels between both *Besnoitia* species have been detected.
  - **Second.** The results have also shown a conservation of the antigen repertoire between both *Besnoitia* species. In particular, 17 abundant and/or immunogenic proteins in both *B. besnoiti* and *B. tarandi* -tachyzoite proteomes have been identified. A total of three proteins related to metabolism, two HSPs, four proteins involved in host cell invasion, one protein involved in cell redox homeostasis, as well as other seven proteins involved in conservative pathways have been identified herein for the first time. Additionally, five spots responsible for cross-reactions between *B. besnoiti* and anti-*N. caninum* specific antibodies have been identified: fructose-1,6-bisphosphate aldolase, ENO, HSP60, HSP90 and actin.
  - **Third.** The difficulty in identifying new *B. besnoiti* diagnostic targets by proteomic approaches has been illustrated. Moreover, the difficulty to differentiate between *B. besnoiti* and *B. tarandi* infections with current serological assays has been also corroborated. Due to absence of the *B. besnoiti* genome sequence, additional approaches are needed to increase the identification of valuable diagnostic targets.

- Sub-objective 3.3
  - **First.** A panel of eight monoclonal antibodies has been developed herein. Among these, the *Besnoitia* spp. tachyzoite specific MABs 2.G.A, 2.A.12 and 2.G.4 arise as promising diagnostic candidates, since cross-reactions with other closely

related Sarcocystidae parasites have not been observed. In addition, MABs 3.10.8, 5.5.11, 1.17.8 and 8.9.2 may also be considered as potential diagnostic targets. However, cross-reactions between these MABs and *N. caninum* should be first discarded. Moreover, the absence of cross-reactions between MABs 1.17.8 and 2.G.A and *B. tarandi* supports their additional value as specific diagnostic markers.

- **Second.** The approach followed here has enhanced the tools available for the study of *Besnoitia* spp. cell biology. The tachyzoite-specific MABs could be employed as markers of tachyzoite-bradyzoite conversion. Two MABs have recognized the surface of *B. besnoiti* tachyzoites, three have recognized the apical tip of the tachyzoites and three have illustrated granular content inside the tachyzoite compatible with dense granule staining.

o Sub-objective 3.4

- **First.** A highly sensitive and specific new ELISA based on lyophilized tachyzoites has been developed and validated under the worst-case scenario. The results derived from the comparison of the new BbSALUVET ELISA 2.0 and the previously developed APure-BbELISA based on an enriched tachyozite membrane extract have demonstrated that both tests can be used indistinctly without the need of confirmatory assays for diagnosis and epidemiological studies. On the other hand, Western blot may still be recommended to confirm PrioCHECK Besnoitia Ab2.0 ELISA results since it showed moderate Se values.
- **Second.** The versatility of BbSALUVET ELISA 2.0 has been demonstrated due to its additional usefulness for the diagnosis of *Besnoitia* spp. infection in wild ruminants.

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